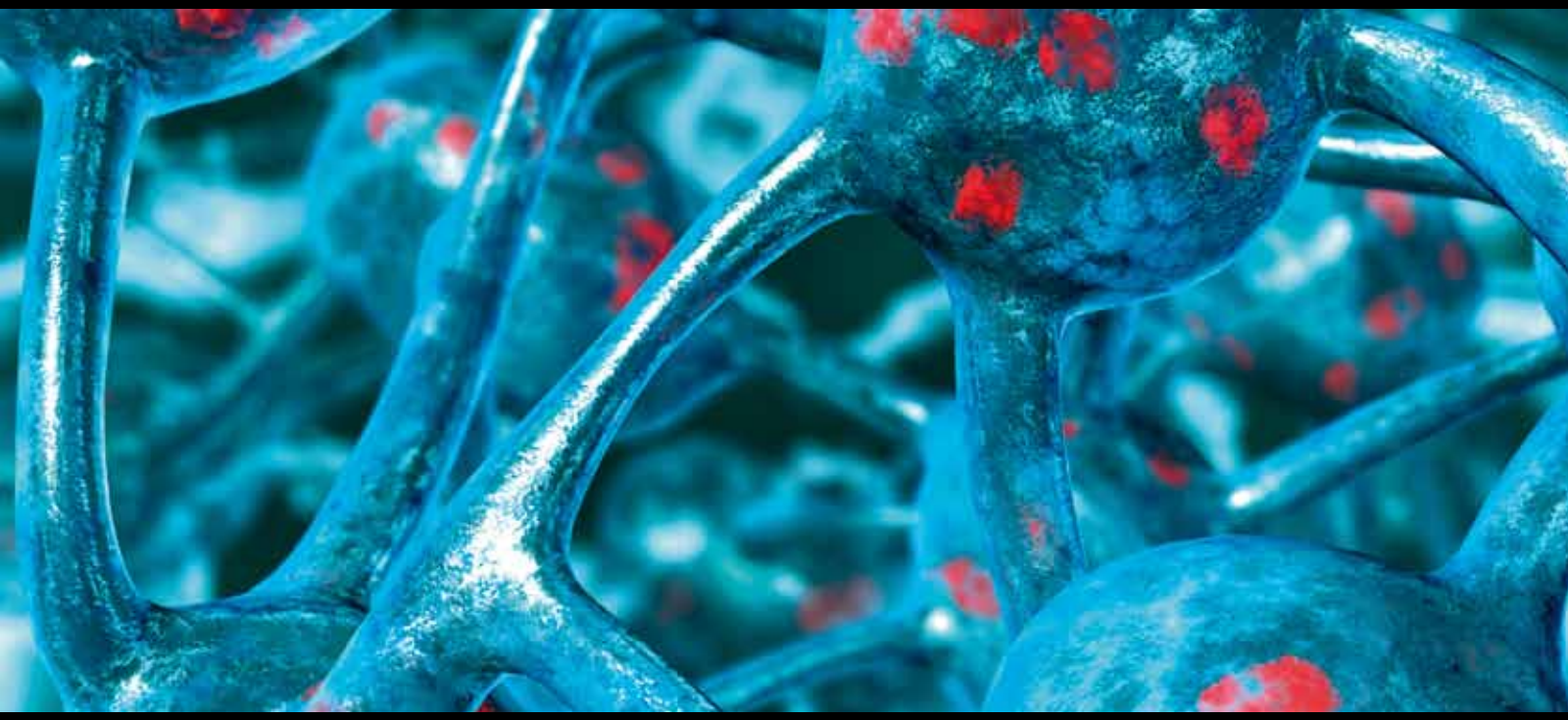
Implication of Autophagy in Parkinson's Disease

Guest Editors: Rosa Ana González-Polo, José Manuel Fuentes, Mireia Niso-Santano, and Lydia Álvarez-Erviti



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Editorial Board

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Editorial

Implication of Autophagy in Parkinson's Disease

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Autophagy is an intracellular catabolic mechanism mediated by lysosomes, which is responsible for most of the degradation and recycling of cytoplasmic components and intracellular dysfunctional or damaged organelles. Increasing evidences suggest that autophagic deregulation causes accumulation of abnormal proteins or damaged organelles, which is a characteristic of chronic neurodegenerative conditions, such as Parkinson's disease (PD), a multifactorial disorder, which is neuropathologically characterized by age-dependent neurodegeneration of dopaminergic neurons in the midbrain. Indeed, promoting the clearance of aggregate-prone proteins via pharmacological induction of autophagy has proved to be an useful mechanism for protecting cells against the toxic effects of these proteins in the context of neurodegenerative diseases and protecting neurons from apoptosis. This special issue is composed of seven excellent reviews addressing the analysis of different models of Parkinsonism in which autophagy plays a key role.

The paper entitled "*Parkinson's disease and autophagy*" by E. Sánchez-Perez et al. reviews some of the mechanisms underlying gene mutations associated with autophagy in PD familial cases. The authors expose as both deficits and stimulation of autophagy underlie with neurodegeneration, suggesting that altered protein and organelle clearance, either by excess or deficit, are involved in the onset of PD. They claim that the mechanisms that could explain this apparently paradoxical behavior are not clear, and further investigation is required in order to use the autophagy machinery and mitochondria and protein-aggregates removal as an effective and safe therapeutic strategy in the treatment of familial and sporadic PD.

The paper "*Dopamine oxidation and autophagy*" by P. Muñoz et al. resumes as aminochrome (a dopamine derevative) has been proposed to play an essential role in the degeneration of dopaminergic neurons containing neuromelanin by inducing autophagy dysfunction in these cells. In this sense, aminochrome is able to induce the formation of α-synuclein protofibrils that inactivate chaperone-mediated autophagy and also the formation of adducts with α- and β-tubulin, which induce the aggregation of the microtubules required for the fusion of autophagy vacuoles and lysosomes. The authors conclude that aminochrome is clearly implicated in the dysfunction of protein degradation in dopaminergic neurons.

In the paper entitled "*Mitochondrial dynamics and mitophagy in the 6-hydroxydopamine preclinical model of Parkinson's disease*", M. F. Galindo et al. discuss the participation of mitochondrial dynamics and autophagy in the 6-hydroxidopamine-induced PD model. They focus your attention on the regulation of dynamic mitochondrial processes such as fusion, fission, and mitophagy, with special emphasis in the role of the second messengers and reactive oxygen species as well as mitochondria as the headquarters of cell death. Finally this paper highlights the therapeutic potential of small-molecule inhibitors of mitochondrial division in PD.

The article "*Methamphetamine and Parkinson's disease*" by N. Granado et al. focuses the role of methamphetamine in PD. This review shows that methamphetamine, an amphetamine-type stimulant which actually is the second most widely used illicit drug in the world, damages dopaminergic neurons in the substantia nigra, resulting in a significant loss of dopamine in the striatum. Biochemical and neuroimaging

studies evidence that molecular changes are similar to those observed in PD patients.

In relation with the previous review, the paper "*N-Acetyl cysteine protects against methamphetamine-induced dopaminergic neurodegeneration via modulation of redox status and autophagy in dopaminergic cells*" by P. C. Shivalingappa et al. reveals that the loss of cellular levels of glutathione is one of the pivotal mechanisms involved in methamphetamine-induced neurotoxicity and autophagy in mesencephalic dopaminergic neuronal cells. They claim that the treatment with N-acetyl cysteine partially reverses methamphetamine-induced apoptotic cell death, possibly by replenishing glutathione levels. Interestingly this paper is the first report demonstrating that N-acetyl cysteine pretreatment can ameliorate methamphetamine-induced autophagy, highlighting the importance of redox status of the cell in methamphetamine-induced dopaminergic neurodegeneration.

J. M. Bravo-San Pedro et al. discuss in the review article entitled "*Parkinson's disease: leucine-rich repeat kinase 2 and autophagy, intimate enemies*" the role of leucine-rich repeat kinase 2 in autophagy and how the deregulations of this degradative mechanism in cells can be implicated in the PD etiology. This review claims that LRRK2 protein is involved in cellular autophagy through direct modulation, the alteration of its own kinase activity, or the mediation of autophagy in response to external stimuli. Therefore, the authors affirm that is important to understand the activity of LRRK2 to elucidate the cellular death that has been identified in studies of *park8* mutations. In this paper the authors conclude that this knowledge would be essential for the development of strategies for reducing the cellular sensitivity and cell death that could trigger the development of PD.

Finally, P. Gómez-Suaga et al. delve into the relationship between autophagy and LRRK2 in the review article entitled "*A link between autophagy and the pathophysiology of LRRK2 in Parkinson's disease*" where they discuss current knowledge about mechanism(s) by which mutant LRRK2 may regulate autophagy, which highlights additional putative therapeutic targets. They claim that currently many questions remain to be addressed, such as whether TPCs (or NAADP binding proteins) are LRRK2 targets, whether LRRK2 causes indeed measurable changes in intracellular calcium levels, or how LRRK2 regulates the activity or localization of distinct rab proteins. In this sense, authors stated that further work would be needed for delineating the precise molecular links between LRRK2, autophagy, and NAADP-mediated events.

The compilation of these reviews included in this special issue of the relationship between PD, and autophagy, shows that there is much controversy in this field. Currently there are many research lines that require a lot of work to get there someday to be able to clarify a possible route of finding treatments against PD based on the mechanism of autophagy.

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Review Article

Methamphetamine and Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disorder predominantly affecting the elderly. The aetiology of the disease is not known, but age and environmental factors play an important role. Although more than a dozen gene mutations associated with familial forms of Parkinson's disease have been described, fewer than 10% of all cases can be explained by genetic abnormalities. The molecular basis of Parkinson's disease is the loss of dopamine in the basal ganglia (caudate/putamen) due to the degeneration of dopaminergic neurons in the substantia nigra, which leads to the motor impairment characteristic of the disease. Methamphetamine is the second most widely used illicit drug in the world. In rodents, methamphetamine exposure damages dopaminergic neurons in the substantia nigra, resulting in a significant loss of dopamine in the striatum. Biochemical and neuroimaging studies in human methamphetamine users have shown decreased levels of dopamine and dopamine transporter as well as prominent microglial activation in the striatum and other areas of the brain, changes similar to those observed in PD patients. Consistent with these similarities, recent epidemiological studies have shown that methamphetamine users are almost twice as likely as non-users to develop PD, despite the fact that methamphetamine abuse and PD have distinct symptomatic profiles.

1. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting an estimated 7 to 10 million people worldwide. Incidence of the disease increases with age. PD usually affects people over the age of 50, but an estimated 4% of PD cases is diagnosed before the age of 50. Early in the course of the disease, the most obvious symptoms are movement-related. These include shaking, rigidity, slowness of movement, and difficulty with walking and gait. Later, cognitive and behavioral problems may arise, with dementia commonly occurring in the advanced stages of the disease. Other symptoms include sensory, sleep, and emotional problems. PD is caused by degeneration of midbrain dopaminergic neurons that project to the striatum. The loss of striatal dopamine is responsible for the major symptoms of the disease. Although a small proportion of cases can be attributed to known genetic factors, most cases of PD are idiopathic. While the aetiology of dopaminergic neuronal demise is elusive, a combination of genetic susceptibilities, age, and environmental factors seems to play a critical role [1]. Dopamine degeneration process in PD involves abnormal protein handling, oxidative stress, mitochondrial dysfunction, excitotoxicity, apoptotic processes, and microglial activation/neuroinflammation.

2. Epidemiology and Pharmacology of Methamphetamine Use

Methamphetamine is an addictive, highly water-soluble CNS (central nervous system) stimulant. It belongs to the group of synthetic drugs chemically related to amphetamine; however, its effects on the CNS are much more pronounced than those of the parent compound. Abuse of these illegal psychostimulants has become an international public health problem, with an estimated 14 to 52 million amphetamine-type stimulant users worldwide, exceeding the total number of cocaine

abusers and second only to the number of cannabis abusers [2]. Hydrochloride methamphetamine, known as "meth" or "speed", can be found in the powder state, compressed into tablets or capsules of 10 to 15 mg, or in a purer crystalline form.

Methamphetamine is taken by abusers for several desired effects: euphoria and a sense of well-being, increased physical activity and energy, and decreased anxiety. These effects appear immediately after drug consumption and can last for several hours. They may be accompanied by acute adverse effects such as increased blood pressure and heart rate, which may cause irreversible damage to blood vessels in the brain, resulting in cerebrovascular accidents, stroke, and death. Methamphetamine also produces hyperthermia, mydriasis (pupil dilation), flushing, tremors, trismus and bruxism, muscle tension, loss of appetite or anorexia, and loss of pleasure in food intake.

Methamphetamine is an addictive drug, and abusers may rapidly develop tolerance. The most common symptoms of chronic methamphetamine abuse are temporomandibular joint syndrome, dental erosion, and myofacial pain [3]. Long-term use also produces lack of appetite, weight loss, accelerated aging, nose-bleeding problems, nonhealing wounds, and tooth decay and fracture known as "Meth mouth". Psychiatric symptoms include anxiety, depression, increased aggression, social isolation, psychosis, mood disturbances, and psychomotor dysfunction. Long periods of high consumption can cause paranoid psychosis. In addition, deficits in attention, working memory, and decision making have been detected in chronic methamphetamine addicts. Withdrawal from methamphetamine can cause irritability, fatigue, impaired social functioning, and intense craving for the drug. There is evidence that the negative neuropsychiatric consequences of methamphetamine abuse are due, at least in part, to drug-induced neuropathological changes in the brain [4].

3. Methamphetamine Toxicity in Experimental Animals

3.1. Methamphetamine Toxicity in the Striatum. Animal studies have shown that methamphetamine can cause long-term dopamine terminal damage as well as dopamine neuronal body loss. In rodents, repeated administration of methamphetamine causes a decrease in dopaminergic markers such as tyrosine hydroxylase (TH) and dopamine transporter (DAT) (see Figure 1), accompanied by a reduction in TH activity, reduced levels of dopamine (DA) and its metabolites (3,4-dihidroxyphenylacetic, DOPAC, homovanillic acid, HVA), and decreased levels of vesicular monoamine transporter 2 (VMAT2). These effects occur primarily in the striatum (caudate-putamen), but as well in the cortex, thalamus, hypothalamus, and hippocampus [5–10]. Methamphetamine induces neurotoxicity in a dose-dependent manner [11] as do other amphetamine-derivatives like MDMA [12, 13]. Although partial recovery of TH and DAT fibers occurs after methamphetamine administration, methamphetamine-induced neurotoxicity is persistent. In mice, the greatest dopaminergic fiber loss is seen 1 day after methamphetamine administration (Figure 1). Neurotoxic effects persist for more than seven days after methamphetamine exposure [5, 14, 15] and one month after MDMA exposure [13]. Drugs that induce parkinsonian symptoms and TH loss such as MPTP in mice also show a partial recovery with time in nonhuman monkeys and mice [16]. The time courses and degrees of TH and DAT fiber recovery after methamphetamine or after MDMA exposure are similar, suggesting terminal regrowth, as these two proteins are independently regulated (Figure 1). In addition, there is partial recovery of dopamine levels in the striatum [5, 7, 12], strongly suggesting that the regrown terminals are functional. The mechanisms responsible for the partial recovery are not known, but might involve compensatory sprouting and branching as has been reported for regrowth following MPTP-induced damage [17]. Dopamine terminal recovery has also been described in rhesus monkeys and velvet monkeys, although it appears to occur on a slower timescale than in mice: methamphetamine-induced dopaminergic damage persists for more than 12 weeks in velvet monkeys and more than 3 years in rhesus monkeys [11, 18], demonstrating the persistence of methamphetamine-induced brain damage.

Interestingly, striatal TH cells that appear in Parkinsonian brains [19] and in 6-OHDA- and MPTP-denervated animals [20, 21] are also evident after methamphetamine treatment (unpublished observations). These TH neurons only appear in severely dopamine-denervated striatal areas and, therefore, represent evidence in support of the strong denervation that methamphetamine use can cause.

3.2. Methamphetamine Toxicity in the Substantia Nigra. In addition to TH fiber loss, methamphetamine administration produces dopamine cell body loss in the substantia nigra pars compacta (SNpc), as indicated by stereological counts in TH-stained SN sections from mice treated with 3 methamphetamine injections (5 mg/kg) at 3-hour intervals. These counts show 20 to 25% dopaminergic cell loss, measured at different time points after methamphetamine exposure. The observed pattern of TH-stained neuron loss is very similar to the pattern of Nissl-stained neuron loss, indicating that neuronal loss is specific to dopaminergic neurons. Dopamine cell body loss was confirmed via staining with Fluoro-Jade, a general marker of neuronal degeneration that fluoresces after administration of known dopaminergic toxins such as 6-OHDA and MPTP [22]. Fluoro-Jade stains scattered neurons degenerated in the SNpc after methamphetamine treatment. It is possible that the lack of complete recovery of TH fibers in the striatum is related to the loss of dopaminergic neurons in the SNpc [5, 7, 15, 23, 24], resembling what occurs in Parkinson's disease [16].

3.3. Neurotoxicity Pattern of Methamphetamine. As in PD, in which the nucleus accumbens is more resistant to dopamine loss than the putamen [25, 26], methamphetamine-induced dopaminergic loss occurs mainly in the nigrostriatal dopaminergic pathway, while the mesolimbic pathway is more resistant [6]. Moreover, the two functional and

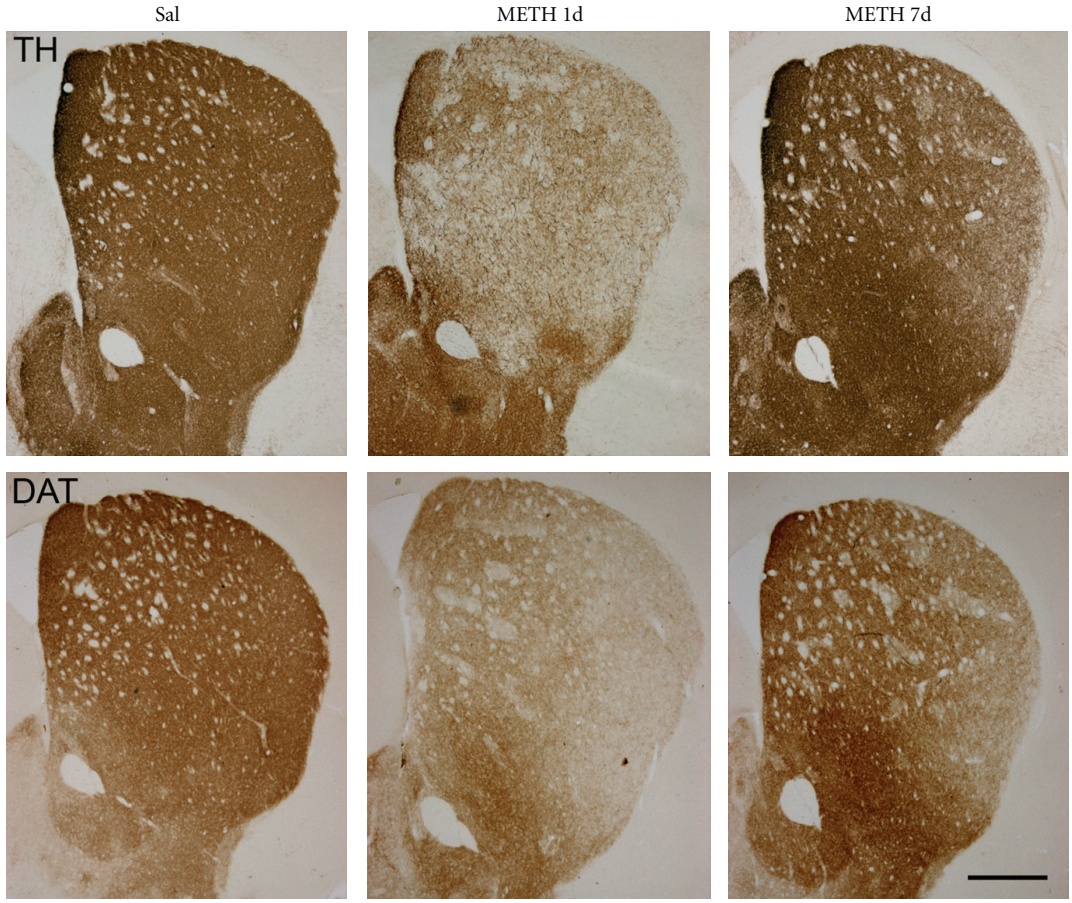

Figure 1: Time-course of TH and DAT fiber lost change after methamphetamine administration. Photomicrographs of striatal sections from mice treated with saline or METH stained for TH and DAT to illustrate the loss (1 day) and the partial recovery (7 days) of dopamine fibers that occur after methamphetamine administration. Animals were killed 1 and 7 days after treatment. Bar indicates 500 μm.

cytoarchitectonic compartments of the striatum, the striosomes and matrix, have different vulnerabilities to methamphetamine. Striosomes, which are connected with the limbic system and functionally associated with reward-related and emotional behaviours [27, 28], are more vulnerable to methamphetamine-induced dopaminergic terminal loss than the matrix (Figure 2; see also [6]), which is connected to sensorimotor regions of the brain closely associated with motor functions [29]. Similarly, greater striatal damage is observed in the striosomes than the matrix in experimental animals following the administration of other neurotoxins such as MDMA [12], MPTP [30], or quinolinic acid [31]. It is also seen in the early stages of Huntington's disease [32] and following ischemia/reperfusion injury [33, 34]. This pattern of neurotoxicity is inversely correlated with SOD (superoxide dismutase) expression in the striatum, suggesting that striosomes, which have lower levels of SOD expression than the matrix, are more vulnerable because they have less antioxidant capacity [6, 12].

3.4. Molecular Mechanisms of Methamphetamine Induced-Neurotoxicity. Although the exact molecular mechanisms of neuronal body loss are not known, there is evidence to suggest the coexistence of different types of cell death, including apoptosis (indicated by the presence of apoptotic- and AIF-positive-cell bodies) and necrosis (indicated by the morphology of neurons stained with hematoxylin-eosin). Increasing evidence demonstrates that methamphetamine and MDMA induce an increase in lipid peroxidation and DNA oxidation as well as increased levels of oxidative stress markers such as hydroxyl radical producing neurotoxicity [35]. Methamphetamine increases expression of nNOS/iNOS (Figure 3) indicating increased synthesis of neuronal nitric oxide [5, 7, 15], which combines with superoxide radicals to form peroxynitrite, a strong oxidant and a major neurotoxin [36]. Induction of nNOS/iNOS by methamphetamine or MDMA (Figure 3) constitutes part of the mechanism of methamphetamine damage, as selective inhibition or genetic inactivation of nNOS and overexpression of cupper zinc superoxide dismutase (CuZnSOD), an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, prevent methamphetamine neurotoxicity [23, 37, 38]. Although methamphetamine increases iNOS expression in the striatum (see Figure 3) [5, 6], there is no basis for supposing the involvement of glial nitric oxide in

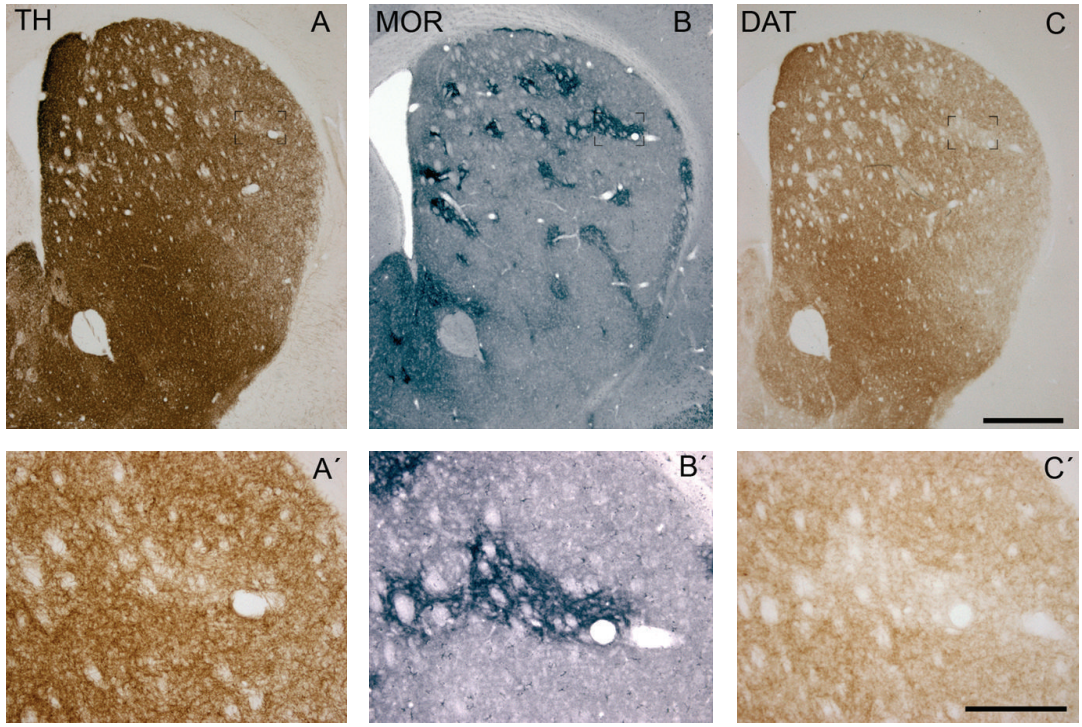

Figure 2: TH- and DAT-ir loss is predominant in striosomes. Serially adjacent sections from a mouse treated with METH stained for TH (A), MOR-1 (B), and DAT (C). Most striatal TH weak patches matched DAT weak patches. These areas corresponded with striosomes as demonstrated by MOR-1 immunostaining. A′–C′ show an example of a striosome at higher magnification. Bar indicates 500 μm (A–C) and 200 μm, (A–C′). Modified from Granado et al. [6].

methamphetamine-induced toxicity, but it is interesting to note that mice deficient in iNOS have increased resistance to methamphetamine-induced dopamine neuron damage [39].

The neurotoxic effects of methamphetamine on the dopaminergic system are accompanied by activation of astroglia and microglia in the same areas [5, 7, 14, 15, 39–41] being strongest in the striatum (Figure 4), the area with biggest toxicity. Glial cells are not activated in the nucleus accumbens, which is not much damaged (Figure 4). In mice, glial activation in striatum and in substantia nigra occurs shortly after methamphetamine administration, as indicated by a significant increase in Mac-1 (a marker of reactive microglia) 24 hours after methamphetamine exposure (Figure 4), and prominent increases in GFAP (a marker of reactive gliosis in response to injury) occur 3–7 days after treatment [5, 15]. The extent of these glial reactions correlates with the observed severity of neurotoxicity [5, 7, 15].

The dopaminergic system is also involved in this toxicity, as demonstrated in various mutant mice in which inactivation of DAT [42], dopamine D1 receptors [5] or D2 receptors [7] affords a significant protection against methamphetamine toxicity [43]. Administration of THC prevents dopaminergic toxicity after MDMA, a similar amphetamine derivative to methamphetamine, by CB1 receptor stimulation which is present in striatal medium spiny neurons [44]. All these receptors are involved in different aspects of learning processes [45–47] that became affected by the chronic use of methamphetamine or MDMA [3, 4, 48, 49].

4. Clinical Toxicology of Methamphetamine

In light of the methamphetamine-induced dopaminergic neurotoxicity and dopamine loss observed in experimental animals, it has been speculated for years that methamphetamine use may predispose consumers to developing neurodegenerative disorders like Parkinson's disease [4, 50, 51]. However, there were no clinical studies proving this hypothesis until recent epidemiological and neuroimaging reports. Neuroimaging studies in humans have started to elucidate the relationship between methamphetamine-abuse and toxicity and susceptibility to neurodegenerative disorders [3, 52].

4.1. Neuroimaging Studies in Human Abusers: PET and MRI Results. Methamphetamine use causes significant long-term dopaminergic neurotoxicity and neurodegeneration in human abusers, and these effects persist long after cessation of drug use. Similar to what has been seen in animal studies, striatal dopamine levels are reduced by ~50% in the brains of human chronic methamphetamine users [52]. Also consistent with animal studies, positron emission tomography (PET) of methamphetamine abusers revealed persistent and significant decreases of 20–30% in dopamine transporter (DAT) in the caudate nucleus and putamen in comparison to control subjects (see Figure 5). This reduction is evident even in abusers who had been detoxified for at least 11 months. Other studies in abstinent former methamphetamine users

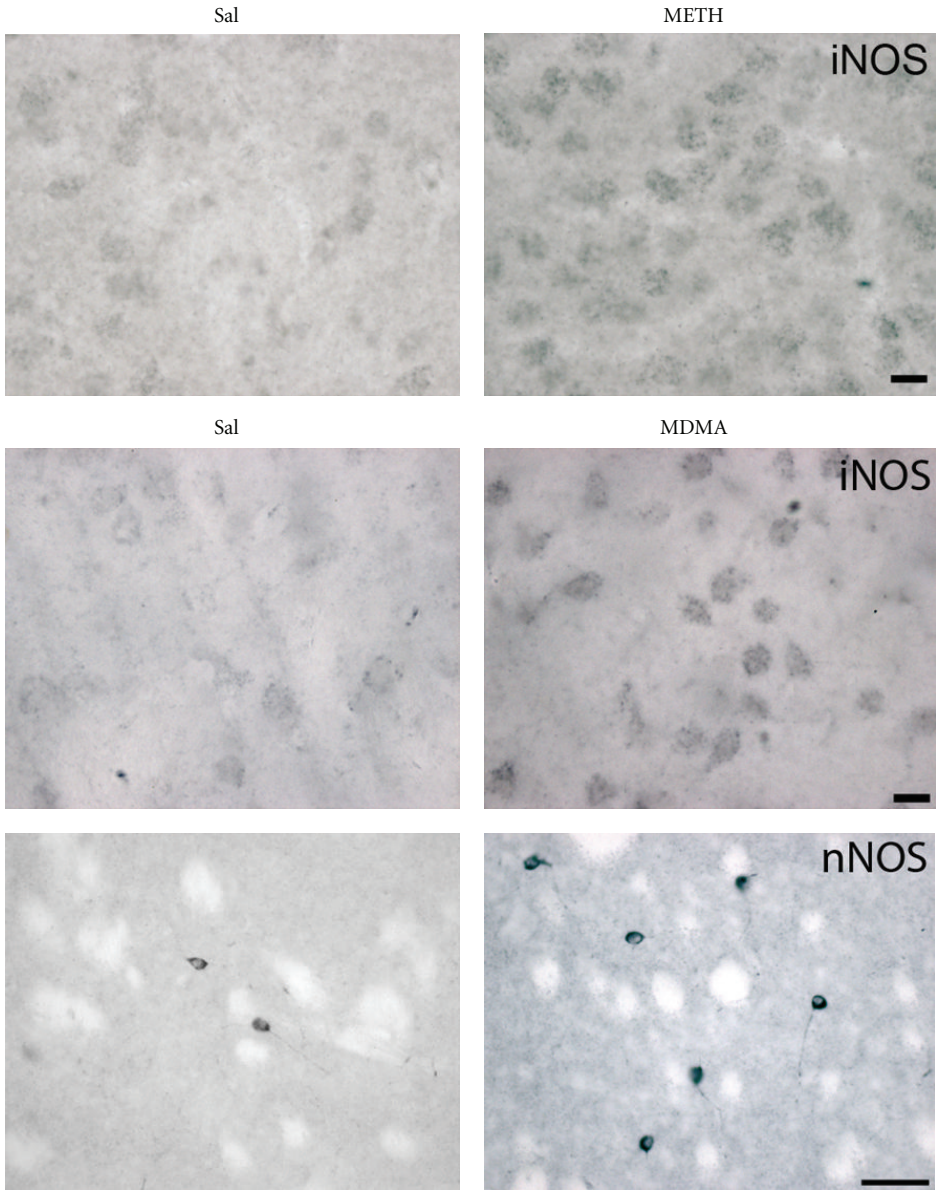

Figure 3: Methamphetamine and MDMA increase the expression of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) in mouse striatum. Photomicrographs of striatal sections of mice treated with saline or methamphetamine (5 mg/kg × 3) or MDMA (20 mg/kg × 3) stained for iNOS and nNOS. Animals were killed 1 day after treatment. Bar indicates 10 μm for iNOS and 50 μm for nNOS. Modified from Granado et al. [13].

have demonstrated reductions in DAT binding densities in the striatum as long as 3 years after methamphetamine withdrawal [53]. This DAT reduction in former addicts has been associated with motor slowing and memory impairment [54–56].

PET studies also found lower densities of serotonin transporter and vesicular monoamine transporter (VMAT2) across striatal subregions, midbrain, and hypothalamus of methamphetamine users [57, 58]. In addition, methamphetamine users exhibited increased levels of the lipid peroxidation products 4-hydroxynonenal and malondialdehyde in the caudate and frontal cortex [59] and increased levels of the antioxidant compounds CuZnSOD and glutathione in the caudate nucleus [60].

PET studies have revealed that human methamphetamine abusers show prominent microglial activation in the midbrain, striatum, thalamus, and orbitofrontal and insular cortices similar to that observed in experimental animals after methamphetamine treatment, with the magnitude of activation inversely correlated to duration of methamphetamine abstinence [61]. Chronic methamphetamine users who died of drug intoxication showed a significant increase in the number of microglial cells in the striatum examined by immunohistochemistry [62]. Intriguingly, several studies

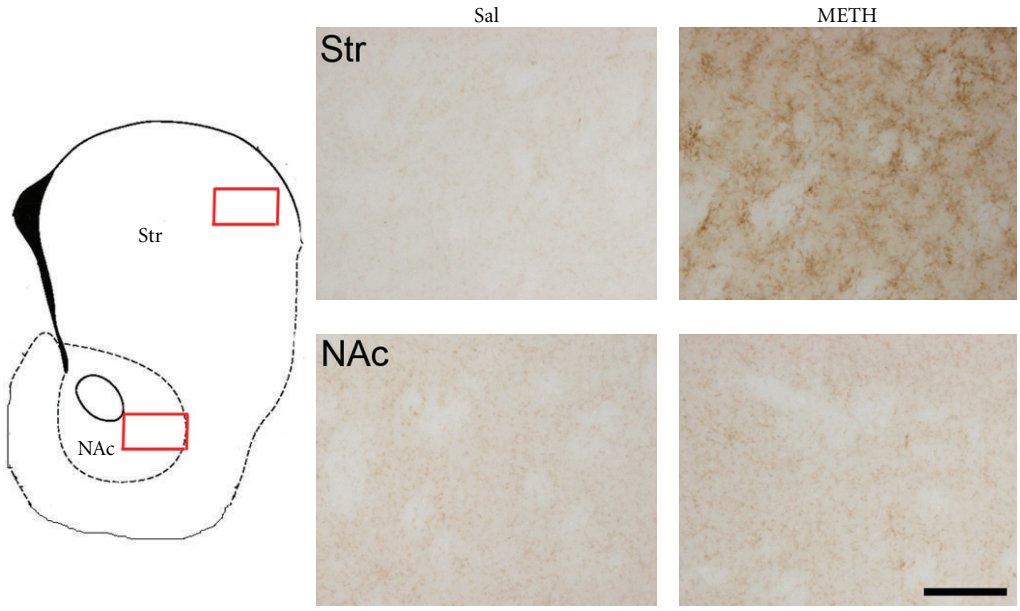

Figure 4: Metamphetamine produces microglial activation in mouse striatum (Str) but not in nucleus accumbens (NAc). Photomicrographs of sections of Str and NAcc of mice treated with saline or metahmphetamine (5 mg/kg × 3) stained for Mac-1. Animals were killed 1 day after methamphetamine treatment for Mac-1. Bar indicates 100 μm.

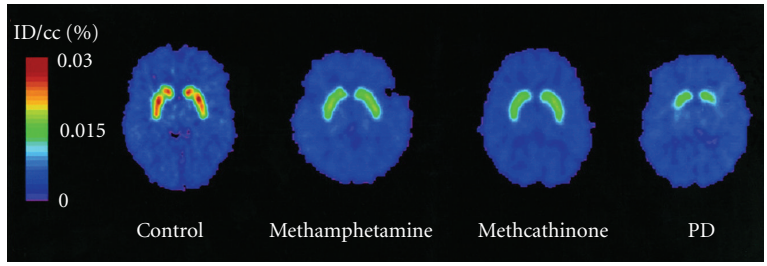

Figure 5: Reduced DAT function in methamphetamine users. PET images showing accumulation of (11C) WIN-35 428 in the striatum in a control subject, an abstinent methamphetamine subject, an abstinent methcathinone subject, and a PD patient 70–90 min after injection of (11C) WIN-35 428. Taken from McCann et al. [53].

have shown that PD patients have more reactive glial cells than do patients without the disease, indicating a possible link between methamphetamine abuse and predisposition to development of PD [63, 64].

Magnetic resonance imaging (MRI) studies demonstrate enlarged striatal volumes in adults who recently abstained from methamphetamine, those with greater cumulative methamphetamine use or longer duration of use, had smaller striatal structures that indicate that the pattern of brain alterations associated with chronic methamphetamine abuse in humans is consistent with cognitive impairment [57]. Moreover, individuals with smaller striatal volumes also performed more poorly on several tests that involved executive function (verbal fluency) and fine motor function (nondominant grooved pegboard). These findings suggest that although methamphetamine use may be associated initially with enlargement of the striatal structures, probably as a compensatory (inflammatory) response, and preserved cognitive function, the volumes of the striatum ultimately decrease with greater methamphetamine usage, accompanied by cognitive impairment. Methamphetamine abusers have increased brain glucose metabolism in the limbic and orbitofrontal regions but relative decreases in the striatum (greater decrease in caudate than in putamen) and in the thalamus [57]. Reductions in DAT levels in the striatum and orbitofrontal and dorsolateral prefrontal cortex have been correlated with the duration of methamphetamine use and the severity of psychiatric symptoms such as anxiety, depression, and psychosis [57, 65]. Furthermore, methamphetamine abusers show severe gray matter decreases in cingulate, limbic, and paralimbic cortices [66] and enlarged striatal volumes [57]. In addition, MR spectroscopy shows reduced concentrations of a marker of neuronal integrity, N-acetylaspartate and total creatine in the basal ganglia [57]. All these findings indicate that methamphetamine abuse is associated with persistent physiologic changes in the human

brain, similar to those seen in experimental animals, and that these changes are accompanied by motor and cognitive deficits [67].

4.2. Motor and Behavioural Deficits in Methamphetamine Abusers. Although the dopaminergic damage seen in methamphetamine abuse and PD is similar, the symptomatology is largely different. None of the symptoms of methamphetamine abuse is similar to the clinical features of Parkinson's disease; thus, there is no symptomatic reason to expect that PD will arise due to drug-induced dysfunction in the dopaminergic system [68]. Although motor deficits have been reported in chronic methamphetamine abusers, these deficits do not typically involve gross movements, as in PD, but rather affect fine motor dexterity, for example, placing pegs in a pegboard [4, 69]. A plausible explanation for this lack of immediate parkinsonian symptomatology was given by Moszczynska et al., [68] who found that in methamphetamine users, mean dopamine levels were more reduced in the caudate (−61%) than in the putamen (−50%), a pattern opposite to that seen in Parkinson's disease [70, 71]. Some methamphetamine users had dopamine levels within the parkinsonian range (up to 97% dopamine loss) in the caudate but not in the putamen. As the putamen and caudate subserve aspects of motor and cognitive function, respectively, the authors suggested that methamphetamine users were not parkinsonian because dopamine levels are not sufficiently decreased in the motor component of the striatum. However, the near-total reduction of dopamine in the caudate could explain reports of cognitive disturbances, sometimes disabling, in some drug users [69].

4.3. Increased Risk of Parkinson's Disease in Methamphetamine Abusers. Recent publications examining the connection between methamphetamine abuse and development of PD indicate a correlation between drug use and later development of the disease. Callaghan et al. [72] reported an increase in incidence of PD in methamphetamine users in an epidemiological investigation based on data from California statewide hospital discharge records. They identified 1,863 methamphetamine users, 9,315 patients hospitalized for appendicitis as a nondrug control group, and 1,720 cocaine users as a drug control group. All subjects were aged at least 50 years, had been hospitalized in California between 1990 and 2000, and had been followed for up to 10 years after discharge. The methamphetamine user group showed an elevated incidence of PD, with a 165% higher risk for development of PD than the patients from the control group. These results have been reproduced later by the same group [73], using a larger- and more-age-diverse group of patients (40,000 people hospitalized for methamphetamine versus 200,000 for appendicitis and 35,000 for cocaine) and a 16-year follow-up period. These two studies are the first to link methamphetamine abuse in young adulthood with development of PD in middle age or later, strongly supporting that methamphetamine use increases the risk for developing PD.

5. Conclusions

In experimental animals, exposure to methamphetamine damages dopaminergic fibres in the striatum and their cell bodies in the substantia nigra, echoing the degeneration pattern observed in human patients with PD. Selective damage to dopaminergic terminals in the striatum has also been observed in human methamphetamine users, although there is no evidence so far that methamphetamine damages dopaminergic cell bodies in the human SNpc. Given these results, it is reasonable to think that methamphetamine use may predispose consumers to future development of PD. This hypothesis has been supported by recent epidemiological work indicating that methamphetamine users have an increased risk of developing PD. This is consistent with the persistent neurotoxic effects of methamphetamine in experimental animals and suggests that methamphetamine use may also produce irreversible loss of dopaminergic neurons in the SNpc of human abusers.

PD is a progressive disorder with a presymptomatic interval; that is, there is a period during which the pathologic process has begun, but the motor signs required for clinical diagnosis are absent [51]. Methamphetamine can reduce dopamine levels in the nigrostriatal system significantly before motor symptoms become evident, which may explain why methamphetamine abusers do not display parkinsonism in the early stages of drug consumption. Given the large number of methamphetamine users worldwide, the relationship between methamphetamine intake and PD could become a vast public health problem in the future.

Further investigation is needed to elucidate the causes and mechanisms of methamphetamine-induced damage. This information will identify mechanisms that might also be involved in pathology of PD and highlight potential new therapeutic strategies for prevention or reduction of dopaminergic neurodegeneration.

Authors' Contribution

N. Granado and S. Ares-Santos have equal contribution.

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Review Article

A Link between Autophagy and the Pathophysiology of LRRK2 in Parkinson's Disease

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Parkinson's disease is a debilitating neurodegenerative disorder, and its molecular etiopathogenesis remains poorly understood. The discovery of monogenic forms has significantly advanced our understanding of the molecular mechanisms underlying PD, as it allows generation of cellular and animal models carrying the mutant gene to define pathological pathways. Mutations in leucine-rich repeat kinase 2 (LRRK2) cause dominantly inherited PD, and variations increase risk, indicating that LRRK2 is an important player in both genetic and sporadic forms of the disease. G2019S, the most prominent pathogenic mutation, maps to the kinase domain and enhances enzymatic activity of LRRK2, which in turn seems to correlate with cytotoxicity. Since kinases are druggable targets, this has raised great hopes that disease-modifying therapies may be developed around modifying LRRK2 enzymatic activity. Apart from cytotoxicity, changes in autophagy have been consistently reported in the context of G2019S mutant LRRK2. Here, we will discuss current knowledge about mechanism(s) by which mutant LRRK2 may regulate autophagy, which highlights additional putative therapeutic targets.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder with symptoms including tremor, rigidity, and postural instability [1]. Autosomal-dominant mutations in leucine-rich repeat kinase 2 (LRRK2) comprise the most common monogenic form of PD [2–5]. LRRK2-associated PD is symptomatically and neurochemically largely indistinguishable from sporadic PD cases [6], even though the reported pleomorphic pathology of mutant LRRK2 carriers differs from the rather classical α-synuclein pathology associated with sporadic PD. Variations in LRRK2 have further been reported to increase risk for sporadic PD [7–9], which implicates LRRK2 in both sporadic and familial forms of the disease. The big advantage of studying the function of a mutated gene product as compared to a sporadic disease is that one can generate cellular and animal models carrying the mutant gene to define pathological pathways. In conjunction with the described enzymatic activity of LRRK2 which may be targeted by select kinase inhibitors [10, 11], this has propelled the protein into the limelight of PD research worldwide. However, to develop disease-modifying or neuroprotective therapies around LRRK2, a clear understanding of its normal and pathological function(s) is required. A link between LRRK2 and aberrant macroautophagy has been consistently observed, and here we review our current knowledge of LRRK2's role in autophagy and lysosomal homeostasis with implications for cell demise in PD.

2. LRRK2 Structure and Cellular Localization

LRRK2 is a large multidomain protein belonging to the ROCO family of proteins which are characterized by the presence of leucine-rich repeats, a Ras of complex (ROC) GTPase domain, a C terminal of ROC (COR) linker region, and a kinase domain [12]. Among the many putative pathogenic variants identified to date, six missense mutations in LRRK2 have been clearly shown to segregate

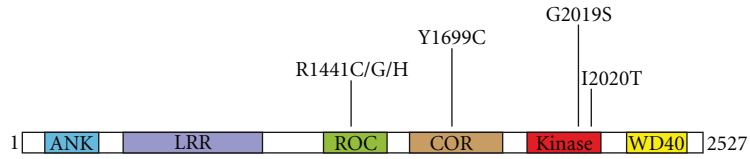

Figure 1: Domain structure and PD mutations of LRRK2. The central region of LRRK2 contains a GTPase domain also called (ROC), a C-terminal of ROC (COR) domain of unknown function, and a kinase domain, flanked on either side by protein-protein interaction domains including an ankyrin repeat domain (ANK), leucine-rich repeats (LRR) and a WD40 domain (WD40). Clearly causative pathogenic mutations are indicated and are clustered around the catalytic domains of LRRK2. Only the G2019S mutation consistently augments kinase activity.

with disease, and thus represent authentic disease-causing variants [13]. Importantly, these mutations all map to the central region comprised of the catalytic domains, indicating that a change in enzymatic activity (either GTPase or kinase) mediates the pathogenic effect(s) of LRRK2 (Figure 1). The G2019S mutation within the kinase domain (Figure 1) is the most frequent pathogenic LRRK2 mutation, having been identified in up to about 40% of familial PD cases dependent on ethnicity, and also detected in apparent sporadic PD cases [4, 5, 7–9]. This mutation has been consistently shown to augment catalytic activity [14], even though the inherent kinase activity of LRRK2 is very low. This may be, at least in part, due to the lack of currently identified and reproducible genuine kinase substrates. LRRK2 kinase is active towards itself [14], and autophosphorylation may represent a physiological readout. The effect of other pathogenic mutations on kinase activity is less clear. Intriguingly, a recent study indicates that the G2385R risk variant causes a partial loss of kinase activity, highlighting the possibility that both too much or too little LRRK2 kinase activity may be detrimental [15]. Mutations in the ROC and COR domain cause a decrease in GTPase, without gross changes in kinase activity [16, 17], suggesting that the GTPase activity may comprise the genuine physiological readout of LRRK2, which may be further modulated by kinase activity [11]. Finally, apart from the catalytic central domains, LRRK2 contains various protein-protein interaction domains including LRRK2-specific, ankyrin, and leucine-rich repeat motifs at the N-terminus, and WD40 repeats near the C-terminus of the protein (Figure 1). The existence of these domains indicates the possibility that it may act as a protein scaffold for the assembly of protein complexes [18]. Indeed, LRRK2 has been reported to interact with a whole array of proteins and may form distinct protein complexes in a cell-type or subcellular compartment-specific manner [19]. In this context, the enzymatic activities of LRRK2 may serve to change the affinity and/or composition of such complexes. Alternatively, a change in enzymatic activity may be the result of a change in protein complex interaction(s). Consistent with the latter possibility, LRRK2 has been reported to exist as a dimer, with dimerization enhancing kinase activity and causing relocalization to intracellular membranes [20–23], even though this has been disputed [24]. In either case, apart from being cytosolic, overexpressed, as well as endogenous, LRRK2 has been reported to localize to specific membrane subdomains including endolysosomal structures in neuronal and non-neuronal cells [25–27]. There, it may interact with and/or regulate distinct protein complexes. Such interactions may be controlled by the catalytic activity of LRRK2, either towards itself or currently unknown substrates. If correct, not only the catalytic activity of LRRK2, but also the modulation of distinct protein interactions should be considered possible targets for therapeutic strategies.

3. LRRK2 and the Regulation of Autophagy

The precise molecular mechanism(s) of LRRK2 function remain unclear. Certain phenotypes are robustly seen, such as the acutely toxic nature of pathogenic mutant forms of LRRK2 upon high-level overexpression in cultured cells [28–31]. Cell death is also evident upon viral vector-mediated expression of mutant LRRK2 *in vivo* [32, 33], and toxicity seems to depend on kinase activity [28, 29, 32]. In neuronal cellular models where cell death is not apparent, neurite shortening represents another consistent phenotype associated with mutant LRRK2 expression [34–42]. Where investigated, this also seems kinase activity-dependent and mediated by macroautophagy [34, 35, 41, 42]. All mutations tested to date have at least one of these effects on cells. Thus, the cellular pathway(s) underlying LRRK2 toxicity may involve altered macroautophagy, which in neurons may lead to neurite shortening and eventual cell demise. If so, elucidating the mechanism(s) by which LRRK2 alters macroautophagy becomes key.

Apart from playing an important role in determining neurite length [43], macroautophagy (thereafter named autophagy) has recently gained attention for its contribution to the pathogenesis of several neurodegenerative diseases including PD [44–46]. Autophagy is a process by which cytosolic constituents, including damaged organelles and aggregated proteins, are engulfed within specialized double-membraned vesicles called autophagosomes. Autophagosomes then fuse with amphisomes or lysosomes, followed by the hydrolytic degradation of products in lysosomes and reformation of these organelles to maintain cellular degradative capacity [47, 48]. Disrupting any part of this process impairs autophagic flux, accompanied by the accumulation of autophagic substrates and organelles [47, 48]. In addition, autophagy and endocytosis share lysosomes as their common end-point [49], such that it has been very difficult to define whether LRRK2 plays positive or negative roles in autophagic-lysosomal clearance.

A wealth of studies indicate that LRRK2 regulates autophagy. For example, various lines of knockout mice have been generated, which display an increase in the number and size of secondary lysosomes and autolysosome-like structures in the kidney [50–52]. An accumulation of lipofuscin granules, highly oxidized, and crosslinked proteins and lipids which cannot be properly degraded, and p62, an autophagy substrate, have also been observed [50–52]. Such abnormal accumulation of undigested material indicates an impairment in the autophagosomal-lysosomal degradation system. To determine a possible defect along the autophagic pathway, the levels of LC3I and LC3II have been analyzed. LC3II, the lipidated form of LC3I, becomes bound to the autophagosomal membrane and serves as a reliable indicator of autophagic activity [53]. Studies analyzing the levels of LC3II in the absence of LRRK2 in the kidney indicate either no change [52], or a biphasic change with an initial enhancement of flux at young age, followed by an impairment of flux over time [50, 51]. This block in flux has been interpreted to be due to an "overload" of the system, resulting in impaired clearance and/or recycling of autophagic components/autolysosomes [51]. Whilst an interesting hypothesis, it depends on assigning a rate-limiting step in the autophagy process, which will need further proof.

In agreement with the *in vivo* data of young animals, RNAi-mediated knockdown of LRRK2 has been found to result in increased autophagic flux under starvation conditions in a human embryonic kidney cell line (HEK293) [25]. Unfortunately, flux experiments were not performed under nutrient-rich conditions in these knockdown cells. Conversely, overexpression of R1441C mutant LRRK2 caused a block in autophagic flux, as evidenced by the accumulation of multivesicular bodies and large autophagosomes containing incompletely degraded material and increased levels of p62 [25]. Similarly, in our studies overexpressing wildtype and G2019S mutant LRRK2 in HEK293 cells, we found improper autophagic-lysosomal clearance, as indicated by an accumulation of autophagic structures and lipid droplets [54, 55]. Thus, at least in the kidney and in kidney-derived cell lines, the normal function of LRRK2 may be related to negatively regulating autophagic clearance and/or lysosomal homeostasis. Too much LRRK2 activity then would dampen, whilst too little activity would enhance autophagic flux. If the latter overloads the system with time, any deregulation of LRRK2 activity may be damaging to the proper functioning of the autophagic pathway *in vivo*.

4. Tissue-Specific versus Universal Regulation of Autophagy

In contrast to kidney, there has been no evidence for the accumulation of autophagic or lysosome-related structures in the brains of aged mice lacking LRRK2 [50–52]. Thus, LRRK2 may perform distinct roles in a tissue-specific manner, with an effect on autophagy in kidney, but not in brain. Alternatively, LRRK1 may functionally compensate for the loss of LRRK2 in the brain, but not in the kidney, the latter of which contains small amounts of LRRK1 versus LRRK2 and thus percentually suffers a much bigger loss of LRRK proteins [56, 57]. In addition, the homo- and heterodimerization of LRRK1 and LRRK2 proteins has been reported [58, 59], with LRRK1 involved in regulating endosomal trafficking [60, 61], consistent with a role for both proteins in recycling and degradation events. Generation of double-knockout lines will be required to delineate whether a complete loss of LRRK proteins in neurons results in age-related changes in autophagy similar to those observed in the kidney.

As another possibility, the overall levels of LRRK proteins present in different tissues may predetermine whether a phenotype is observed upon knockout versus overexpression conditions. For example, as LRRK levels are very high in kidney [56, 57], a knockout strategy may be more adequate to uncover the (normal) role of LRRK2 in autophagic-lysosomal clearance. Conversely, given the low levels of LRRK2 in the brain, an overexpression approach, especially of mutant, hyperactive LRRK2, may be more effective.

Apart from differences in the levels of LRRK proteins, the rate of basal autophagy also displays large differences across distinct tissues. Thus, the same pathogenic mutation of LRRK2 may give rise to different degrees of pathology depending on the cellular milieu in which it is operating [19]. As basal autophagy is very high in the kidney, a deregulation may be more pronounced in this organ as compared to other tissues. Nevertheless, if LRRK2 is a universal modulator of autophagic/lysosomal clearance, changes should also be detectable in other tissues such as brain, albeit possibly to a lesser degree or in an age-dependent manner difficult to track using rodent models. In agreement with a universal role in regulating autophagy, an overexpression approach using G2019S mutant LRRK2 has been reported to cause abnormal accumulation of autophagic and lysosomal structures in primary cortical neurons and neuronal cell lines in culture [34, 35]. Similarly, an accumulation of autophagic vacuoles, including early and late autophagosomes, has been described in the soma and processes in the cortex and striatum from G2019S, and to a lesser degree R1441C, transgenic mice with advanced age [40]. Thus, both *in vitro* and *in vivo*, overexpression of mutant LRRK2 seems to cause impaired autophagic-lysosomal clearance in neurons as well. A decrease in autophagic flux, concomitant with an increase in p62 levels, autophagosomes and lipid droplets has recently also been described in human dopaminergic neurons derived from induced pluripotent stem cells from G2019S mutant LRRK2, but not control patients, after long-term culture [42]. These data are important, as they indicate that endogenous levels of mutant LRRK2 are sufficient to induce an autophagic-lysosomal phenotype in dopaminergic neurons with time. In contrast, fibroblasts from those same patients do not reveal differences in autophagic clearance, consistent with their extremely low levels of basal autophagic activity [42]. However, the latter findings are in contrast to a recent report suggesting elevated levels of autophagic activity [62], and the precise role for mutant LRRK2 in autophagy regulation in fibroblasts remains to be determined. Finally, bone marrow-derived macrophages from mutant LRRK2 mice display a decrease in LC3II levels, possibly highlighting

an autophagic phenotype in those cells as well [63]. Alltogether, the currently available data indicate that LRRK2 can regulate autophagic-lysosomal clearance in neurons as well as a variety of other cell types, possibly in a manner dependent on the basal level of autophagy.

5. Mechanism of Autophagy Regulation by LRRK2

If LRRK2 indeed regulates autophagic clearance, understanding the mechanism of action becomes important to develop alternative and/or complementary treatment strategies. The effects of LRRK2 on autophagic-lysosomal clearance may reflect its primary mechanism of action or may occur secondarily, elicited as a response to some upstream event(s). Even if direct, many distinct scenarios remain possible, as autophagy intersects with both secretory and endocytic pathways at several points [64]. Given its heterodimerization with LRRK1 [58, 59], which has been reported to regulate trafficking events of the epidermal growth factor receptor (EGFR) between early and late endosomes, endosome motility and sorting of the epidermal growth factor receptor (EGFR) to the inner vesicles of multivesicular bodies [60, 61], one may speculate that LRRK2 regulates similar events, with consequences for autophagic pathways involving multivesicular bodies [65].

Apart from this mere analogy, LRRK2 has been shown to interact with the GTPase rab5b, a key regulator of early endocytic vesicle trafficking [66]. Overexpression or knockdown of LRRK2 cause a decrease in presynaptic vesicle endocytosis rates, again indicating that both too much and too little LRRK2 adversely alter the balance of homeostatic mechanisms, in this case controlling endocytosis [66]. Similarly, both overexpression or knockdown of LRRK2 induce defects in vesicle endocytosis upon depolarization of primary neuronal cultures [67, 68], which may involve interactions of LRRK2 with a series of endocytic proteins apart from rab5b [68], but further studies are needed to determine how LRRK2 may regulate the function of any of these proteins. Interestingly, rab5b, apart from regulating the endocytic pathway [69] has recently been shown to play an additional positive role in autophagy by regulating an early step of autophagosome formation in a TORC1-independent manner [70]. Thus, a LRRK2-mediated regulation of rab5b may rather directly impact upon autophagic flux. Indirect LRRK2-mediated regulation of autophagy via changes in endocytosis can be envisioned as well, as endocytosis enables the formation of distinct signal transduction complexes which define specialized endosomal-lysosomal signaling platforms [71]. LRRK2-mediated changes in endocytosis may modulate the formation of those intracellular complexes to regulate signalling cascades including Wnt or MAP kinase cascades [71], both of which have been shown to be affected by LRRK2 [18], and which then may modulate the function of downstream autophagic components.

Multiple data support the idea that LRRK2 also modulates late steps in the autophagic-lysosomal clearance pathway. The fusion of both autophagosomes and endosomes with lysosomes requires rab7, as does the process of lysosome reformation [49, 72–74], and interfering with rab7 function will thus affect autophagic-lysosomal clearance. Indeed, at least in Drosophila, the LRRK2 homolog seems to interact with rab7 on late endosomes and lysosomes to negatively regulate rab7-dependent perinuclear lysosomal positioning required for the efficient degradation of autophagosomes [75]. Another recent study in C. elegans expressing human wildtype or mutant LRRK2 in conjunction with proteostatic stress indicates increased expression of numerous proteins including a subunit of the V-type proton ATPase [76, 77], and the behavioural motor deficits observed in these double-transgenic worms can be reverted by increasing autophagic flux using a rapamycin analog. These data are consistent with our findings that mutant LRRK2 may increase lysosomal pH and concomitantly decrease lysosomal clearance, a process reverted by rapamycin, but not by other compounds which increase autophagy in an mTOR-independent manner [54]. It remains to be seen whether the beneficial effect of the rapamycin analog on motor output is related to an mTOR-dependent increase in degradative capacity as autophagic flux is enhanced, a decrease in protein synthesis, an effect on lysosomal homeostasis, or a combination thereof. Taken alltogether, a picture is emerging whereby LRRK2 may regulate both early and late steps of autophagic-lysosomal clearance in a rab protein-dependent manner (Figure 2).

6. A Link between LRRK2, Autophagy, and NAADP-Mediated Endolysosomal Calcium Signaling

In agreement with other reports, we also found an increase in autophagosome numbers upon transient overexpression of wildtype and G2019S-mutant, but not kinase-dead LRRK2 in various cell lines including dopaminergic neuroendocrine cells [54, 55]. Interestingly, we found that these effects were inhibited by the calcium chelator BAPTA, suggesting that they were calcium-dependent. The effects of LRRK2 overexpression on autophagosome numbers were also blocked when genetically depleting ER calcium stores and were accompanied by an increase in the pH of a population of lysosomes and an increase in the number of lipid droplets. This phenotype closely matches the one triggered by NAADP, which evokes cytosolic calcium signals that can be amplified by ER calcium stores, causes partial alkalinization of acidic stores, and induces lipid accumulation [78–80]. NAADP is a potent agonist-generated second messenger and capable of triggering complex calcium signals which are initiated from acidic stores and are being subsequently amplified by ER calcium release channels [81–83]. Targets for NAADP are likely comprised of the endolysosomal two-pore channels TPC1 and TPC2 [84–86], even though recent studies indicate that NAADP does not directly bind to TPCs, but rather indirectly through currently unidentified associated low-molecular weight binding proteins [87]. In either case, there is a growing appreciation of the importance of endolysosomal organelles as mobilizable calcium stores [81, 88], and intraluminal calcium seems required for endolysosomal

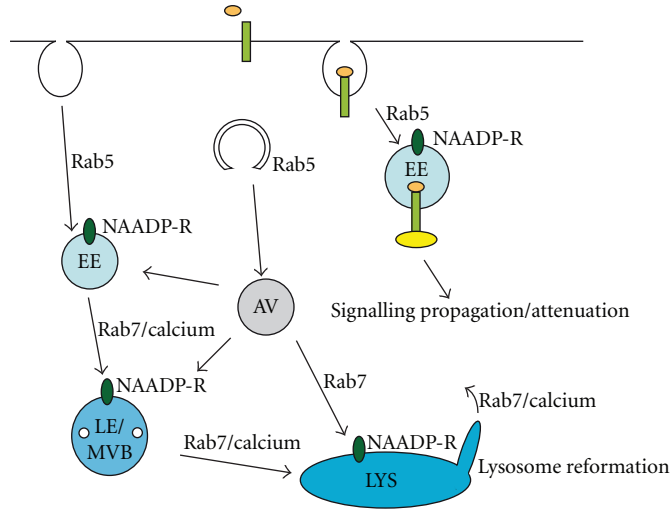

Figure 2: Possible mechanisms by which LRRK2 may regulate events related to endolysosomal and autophagic function. Modulation of rab5 function could cause changes in endocytosis and/or autophagosome formation. Altered endocytosis could also modulate signalling events occurring at the plasma membrane or on intracellular organelles, thereby, indirectly impacting upon autophagy through phosphorylation events of distinct proteins required for the process. At later stages, through modulating rab7 function, LRRK2 may alter the fusion of autophagosomes/endosomes with lysosomes or impair lysosome reformation, which would impact upon autophagic-lysosomal clearance in both cases. As most of the abovementioned membrane fusion/reformation steps require intraluminal calcium, LRRK2 may further regulate endolysosomal clearance by modulating NAADP-sensitive calcium channels (NAADP-R) located on endosomes and lysosomes. The increasing intraluminal calcium concentrations along the endocytic/lysosomal pathway are indicated by the progressively darkened blue color. Ligand binding to receptors, followed by endocytosis and interaction with signalling complexes are schematically indicated. EE: early endosome; AV: autophagosome; LE/MVB: late endosome/multivesicular body; LYS: lysosome. For further details and references, see text.

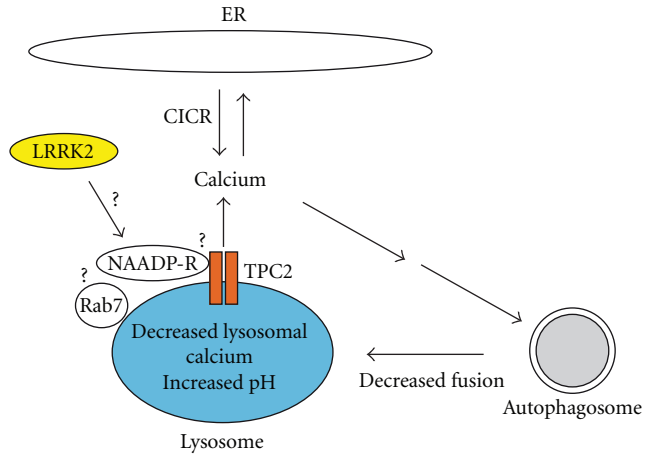

Figure 3: Diagram of proposed mechanism(s) by which LRRK2 regulates autophagy via modulation of NAADP-dependent calcium channels (NAADP-R) on lysosomes. LRRK2 localizes to lysosomes and regulates calcium release through two-pore channels (TPCs). Whether this is due to a direct interaction of LRRK2 with NAADP-R, an indirect interaction via rab7 or additional proteins, or whether it is mediated by a phosphorylation event remains to be determined. Calcium release from acidic organelles then causes calcium-induced calcium release (CICR) from the ER to amplify cytosolic calcium signals, which leads to the activation of a cascade to increase autophagosome numbers. Diminished luminal calcium will further cause a decrease in autophagosome-lysosome fusion, and increased pH may have additional effects on eventually impairing lysosomal proteolysis, leading to the observed autophagic-lysosomal clearance phenotype.

membrane fusion events, thus, directly impacting upon endosomal and autophagic trafficking events [73].

The analogy between the effects of LRRK2 overexpression and NAADP action prompted us to test the connection between NAADP and LRRK2 action. Accordingly, we found that elevation of cellular NAADP levels using a cell permeable NAADP analogue (NAADP-AM) [89] increases autophagosome numbers, lysosomal pH, and lipid droplet numbers, thus, largely mimicking the effects observed upon LRRK2 overexpression [54]. Conversely, the NAADP

antagonist NED19 recently identified by virtual screening methods [90] reverted the effects of LRRK2. The increase in autophagosome number could also be blocked by overexpression of TPC2 mutated within the pore region [91]. This inactive mutant likely acts in a dominant manner similar to TPC1 in which the corresponding residue is mutated [84, 92]. Together, these data uncover a hitherto unknown link between NAADP and LRRK2 function (Figure 3).

7. Summary

A wealth of recent data supports the idea that LRRK2 regulates autophagy. Another ROCO protein family member, death-associated protein kinase 1 (DAPK1), also seems to be an essential regulator of autophagy [93], and it will be interesting to determine whether other ROCO proteins are autophagy modulators as well. Furthermore, LRRK2 variants have been associated with Crohn's disease (CD), an inflammatory bowel disease [94]. As other CD-associated risk genes are also linked to autophagy triggered as an antibacterial response, the disease may result from ineffective control of bacterial infection and resultant chronic inflammation [95]. Similarly, recent data suggest that LRRK2 dysfunction in PD may involve the immune system [96], and the involvement of aberrant autophagy in such process warrants further investigation. Whilst the link between LRRK2 and autophagy is becoming solid, the precise underlying mechanism(s) remain unknown. Both direct and indirect scenarios can be envisioned, and evidence for both is emerging. Rab proteins and calcium seem to play potentially important and not mutually exclusive roles. Calcium is known to both positively and negatively regulate autophagy, and these dual effects may depend on the precise intraorganellar location at which it is required for autophagosome-lysosome or endosome-lysosome fusion, respectively [49, 72]. Many questions remain to be addressed, such as whether TPCs (or NAADP binding proteins) are LRRK2 targets, whether LRRK2 causes indeed measurable changes in intracellular calcium levels, or how LRRK2 regulates the activity or localization of distinct rab proteins. Additional work is needed toward delineating the precise molecular links between LRRK2, autophagy, and NAADP-mediated events.

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Review Article

Parkinson's Disease and Autophagy

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It is generally accepted that a correlation between neurodegenerative disease and protein aggregation in the brain exists; however, a causal relationship has not been elucidated. In neurons, failure of autophagy may result in the accumulation of aggregate-prone proteins and subsequent neurodegeneration. Thus, pharmacological induction of autophagy to enhance the clearance of intracytoplasmic aggregate-prone proteins has been considered as a therapeutic strategy to ameliorate pathology in cell and animal models of neurodegenerative disorders. However, autophagy has also been found to be a factor in the onset of these diseases, which raises the question of whether autophagy induction is an effective therapeutic strategy, or, on the contrary, can result in cell death. In this paper, we will first describe the autophagic machinery, and we will consider the literature to discuss the neuroprotective effects of autophagy.

1. Introduction

Autophagy was initially reported more than 40 years ago [1]. It is a physiological process by which cells remove damaged proteins and organelles through lysosomal degradation. This system prevents the accumulation of products that are not only useless, but potentially toxic. In neurons, this process is considered particularly important since neurons do not replicate; therefore, eventual damaging proteins will not be diluted in subsequent divisions. Autophagy is distinctly regulated in neuronal and nonneuronal cells [2, 3], and recent studies have linked autophagic pathways to several pathological conditions ranging from cancer to neurodegenerative disorders [3, 4]. Moreover, impairment of basal autophagy results in neuronal death [5, 6]. Interestingly, accumulation of proteins is a common feature in several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). In AD, hyperphosphorylated tau-containing neurofibrillar tangles and Aβ deposits are found; in PD, aggregated α-synuclein is a major component in the Lewy bodies; in HD, N-terminal fragments of mutant huntingtin protein (Htt) are found in intracellular inclusion bodies. These findings led to hypothesize that alterations in the autophagic process were responsible for the aggregation of these toxic proteins and consequently to the onset of disease. According to this idea, several reports document an amelioration of toxicity with removal of accumulation of aggregates (for review see [7]). However, other reports challenge this view and suggest that aggregation of toxic products is not correlated with the degree of neurodegeneration; therefore, protein aggregates are considered an epiphenomenon of the disease, not an underlying factor [8–10]. The literature provides enough evidence to feed controversy; in this paper, we will review the data related to the effects of autophagy on neuroprotection, in particular in connection with PD.

2. Autophagy Classification

Based on how the proteins reach the lysosome, autophagy can be classified as (i) macroautophagy, (ii) microautophagy

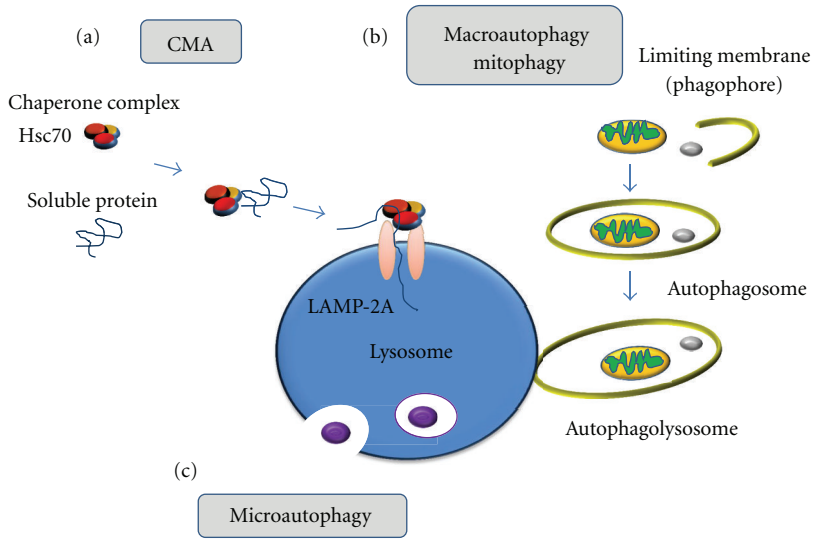

Figure 1: Schematic depiction of the three types of autophagy. (a) Chaperone-mediated autophagy. The cytosolic chaperone protein HSC 70 binds to the substrate protein; the consensus sequence LysPheGluArgGln of the substrate-chaperone complex is recognized by LAMP-2A, a lysosomal membrane receptor. The protein substrate is then unfolded and translocated across the lysosomal membrane to be degraded inside the lysosome. (b) Macroautophagy. Cytosolic material is sequestered by an expanding membrane sac (phagophore) forming a double-membrane vesicle, an autophagosome. Fusion of the autophagosome to the lysosome will expose the content of the autophagosome to lysosomal hydrolases. (c) Microautophagy. Small proteins can be engulfed directly by the lysosome without intermediate vesicles.

and (iii) chaperone-mediated autophagy CMA (Figure 1) [11].

(i) Macroautophagy, usually identified simply as autophagy, is the strategy commonly used for bulk degradation of cytoplasmic proteins and organelles (including dysfunctional mitochondria, which sometimes are referred as mitophagy). It is generally considered to be a nonspecific process in organisms from yeast to humans (with exceptions) and is a multistep process, where the formation of the double-membrane autophagic vacuoles (AVs) or autophagosomes occurs first. These vesicles surround the organelles or proteins to be degraded [12] and later fuse with endosomes to form an intermediate type of vesicle (amphisomes), or directly to lysosomes (autophagolysosomes), where the content will be finally degraded [4]. Macroautophagy can also be induced under conditions of physiological stress, like starvation [13]. The proteins regulating the whole process are autophagy-related proteins (Atg in yeast, ATG in mammals) which were discovered in yeast and have been found highly conserved. Up to date, more than 30 Atg proteins in mammals are known to participate in this intricate process (for review [11]).

(ii) Microautophagy is a much simpler process and occurs when lysosomes engulf cytosolic components directly by membrane involution [14, 15].

(iii) Finally, chaperone-mediated autophagy (CMA) incorporates cytosolic proteins that are brought by chaperones to the lysosome membrane (for review [16]). All the CMA substrates described so far are soluble cytosolic proteins containing a consensus sequence Lys-Phe-Glu-Arg-Gln (KFERQ) [17]. This motif (present in approximately 30% of cytosolic proteins) is recognized by a cytosolic chaperone, heat-shock cognate protein 70 (Hsc70), which transfers protein substrates to the lysosomal membrane, and there, through binding to the receptor lysosome-associated membrane protein-2A (LAMP-2A), they are translocated into the lysosome.

2.1. Autophagy versus Proteasome-Mediated Protein Degradation. Proteasomes are barrel-shaped protein complexes that mainly degrade small, short-lived nuclear and cytosolic proteins [18]. The ubiquitin-proteasome system is also important for the degradation of misfolded proteins in the endoplasmic reticulum [19]. Most proteins are targeted for proteasomal degradation after being covalently modified with ubiquitin. However, substrates need to be unfolded to pass through the narrow pore of the proteasome barrel, which hinders the clearance of oligomeric and aggregated proteins [20]. Under normal circumstances, the ubiquitin-proteasome system is more efficient than basal levels of macroautophagy, so for proteins that have access to both pathways, proteasomes are the favored clearance route. However, when a cytosolic protein is susceptible of aggregation, and therefore a poor proteasome substrate, macroautophagy will become the dominant clearance route [21]. This suggests that dependence of proteins on the macroautophagy pathway for their clearance correlates with their propensity to aggregate [22]. On the other hand, impairment of proteasome pathways has been associated with PD [23, 24] and HD [25]. Moreover, systemic exposure to proteasome inhibitors induces a model of Parkinson [23]. Therefore, it is not surprising that one of the most studied genes associated with

familial PD was parkin, which encodes for an Ubiquitin-protein ligase [26]. However, whether proteasomal impairment is a key stepin familial or sporadic PD in which there are no primary defects in the ubiquitin-proteasome pathway is still not clear.

3. Proteasome and Protein Aggregates: Macroautophagy

Maybe as a consequence of proteasome impairment, or other reasons in familial forms of PD, aggregates of α-synuclein forming the characteristic Lewy bodies (LB) are found. Furthermore, mutant forms of α-synuclein are strongly dependent on the macroautophagy pathway [22, 27]. Confirming these findings, it has been shown that inhibition of macroautophagy has much smaller effects (if any effect at all) on the clearance of wild-type α-synuclein than on the clearance of the mutant aggregate-prone species [27]. This is also the case for other aggregates such as Htt in HD [28, 29].

Beclin-1 (a mammalian homologue of ATG6) is required for the formation of the autophagosome; alterations in beclin-1 have been linked to PD. Mutations in the PTEN-induced putative kinase 1 (PINK1) gene also cause autosomal recessive PD. The full-length PINK1 interacts with Beclin1, and the overexpression of PINK1 significantly enhances both basal and starvation-induced autophagy, which can be reduced by beclin1 gene knockdown. On the other hand, when a lentivirus expressing beclin-1 was delivered to the brain of α-synuclein transgenic mouse, enhanced lysosomal activation and reduction of accumulation of α-synuclein were observed [30]. However, overexpression of both mutant and wild type α-synuclein may also be accompanied by the induction of macroautophagy [31]. Moreover, functional deficiency of DJ-1 (associated with familiar forms of PD), and mutant forms of LRRK2 (leucine-rich repeat kinase 2, also linked to PD), lead to increased autophagy in murine and human cells [32] and in transfected cells [33].

Although neuronal autophagy appears primarily to be a protective process in the nervous system, it can also play a paradoxical role in neuronal death. With respect to the role of autophagy in neuronal death, several studies employing PD toxins, a mutant familial PD gene, and postmortem PD brains have demonstrated an important role for autophagy in promoting the death of dopamine neurons. For example, autophagic cell death has been observed in nigral dopamine neurons of PD patients [34]. MPP+ or dopamine toxicity-induced oxidative stress increases the number of AVs, autophagy, and cell death, all of which differs from what is observed in starvation-induced autophagy [35]. These studies suggest that pathogenic autophagy associated with neuronal death occurs and may be distinct from basal neuronal autophagy. The contribution of autophagy and autophagic cell death to degeneration of dopamine neurons may vary depending on the initial cause and specific cellular context [36]. A better understanding of autophagic stress and further identification of autophagic cell death mechanisms may lead to therapeutics that help restore homeostasis to dopamine neurons in PD.

4. Chaperone-Mediated Autophagy

Several of the 10 genes known to be mutated in association with PD encode proteins with sequences compatible with the CMA-targeting motif [16]. α-synuclein is degraded by macroautophagy (as discussed earlier) but also by CMA [27, 37]. Interestingly, it has been reported that mutant α-synuclein cannot be degraded by CMA, but, in addition, it seems to act as a blocker for other proteins using this pathway. Moreover, in sporadic Parkinson, where no mutations of α-synuclein are found, dopamine adducts of α-synuclein [38] behaved like the mutant protein, that is inhibiting cellular CMA process [39]. Other proteins like the myocyte-specific enhancer transcription factor 2D (MEF2D), that is, a *bona fide* CMA substrate [40], have been observed to increase their cytosolic levels in mice models of PD, in PD patients [41] and in neurons with partial blockage of CMA [40]. In these reports, blockage of CMA process seems to be a causal factor in the onset of PD.

5. Mitocondria ROS/RNS and Autophagy

Mitochondrion is the major source of ATP in the cell; this energy is obtained via a multistep process where carbons atoms are oxidized to CO_2. Damaged mitochondria are accumulated, and that contributes to inefficient oxygen reduction. As a result, highly reactive species both oxygen and nitrogen derived (ROS/RNS) are formed. Defective mitochondria are not the only source of ROS and RNS, but, regardless of their source, reactive species can in turn target mitochondria (Figure 2). Cells have efficient systems to detoxify ROS and RNS. When there is an excess of reactive species due to altered balance in the production and removal of ROS/RNS, pathological conditions such as PD and other neurodegenerative diseases associated occur [42–44]. It is generally accepted that autophagy is responsible for diminishing ROS/RNS damage, but, given the variety of reactive molecules and their location, it is yet not clear whether this is the case in every situation. Specific forms of ROS and RNS include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), nitric oxide (NO), and peroxynitrite ($ONOO^-$). Lipid peroxidation is a consistent feature of neurodegenerative diseases, and biologically active RLS, such as HNE (4-hydroxynonenal), accumulates in brains of patients with PD and AD [45–47].

The aggressiveness of these molecules makes them toxic to the cells; they react with proteins and lipids, inactivating them or making them prone to aggregation. For instance, α-synuclein and parkin have been found to be S-nitrosylated (addition of NO to thiol groups) in relation to PD [48]. Nitrogen modified α-synuclein makes the protein prone to aggregation [49, 50], and S-nitrosylation of parkin inactivates it [51]. It was recently shown that parkin is selectively recruited to damage mitochondria by PINK1, a mitochondrial serine/threonine kinase, and another recessive autosomal mutated gene linked to inherited forms of PD. PINK1 is usually present at low levels on the mitochondrial membrane [52]. When the mitochondrial membrane potential is dissipated, full-length PINK1 is accumulated in the outer mitochondrial membrane. Thus, damage to mitochondria

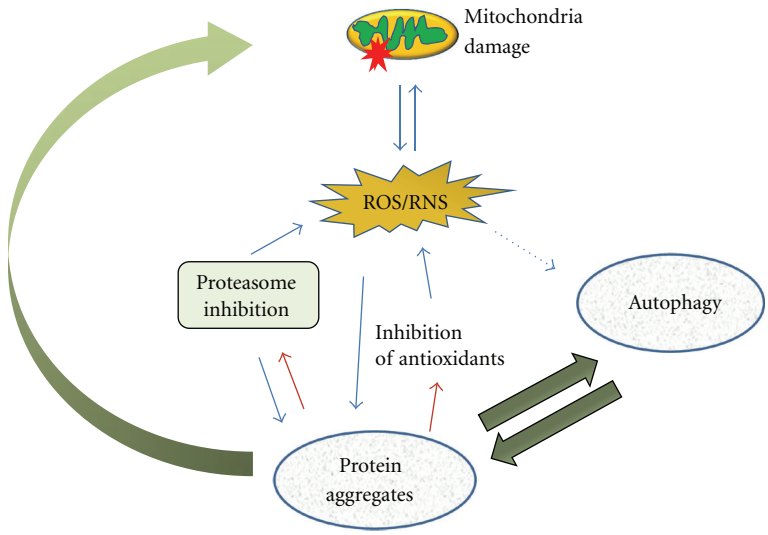

Figure 2: ROS/RNS production as a result of defective mitochondria respiratory activity can be induced by a number of factors like protein aggregates. Reactive species can also be generated by other cellular oxidases. These ROS/RNS species can modify several proteins which can stimulate and/or inhibit autophagy. In addition, reactive species produced or not in the mitochondria can target this organelle and induce further damage to it.

facilitates the rapid accumulation of PINK1, and, subsequent to it, parkin is recruited to the mitochondria to induce mitophagy [53]. This discovery revealed a link between the mitochondrial quality control and proteins mutated in familial PD. Moreover, it further implicates a failure to eliminate dysfunctional mitochondria in the pathogenesis of PD. In addition, the voltage-dependent anion channel 1 (VDAC1) is a target for parkin-mediated polyubiquitination of Lys 27 and mitophagy [54]. Thus, pathogenic parkin mutations, together with PINK1 mutations, could lead to the disruption of mitochondrial recruitment of parkin, ubiquitination of mitochondrial substrates, formation of AVs, and the final clearance of damaged mitochondria *via* mitophagy. This putative role of PINK1 as a "guardian of mitochondrial integrity" seems to be confirmed by reports of PINK1 and parkin knockout models, where an accumulation of damaged mitochondria in various tissues including dopamine neurons occurs [55, 56]. Moreover, α-synuclein also targets to mitochondria, where it causes a decrease in complex I activity and/or mitochondrial damage [57, 58]. This mitochondrial damage causes an increase in mitophagy, presumably as an attempt to clear damaged mitochondria [59]. If mitophagy was not adequate for clearance of dysfunctional mitochondria, these deficiencies could contribute to cell death and neurodegeneration [60–62]. Altogether, these observations suggest that neuronal autophagy is essential for the turnover of damaged mitochondria and that the failure to induce mitophagy may underlie the selective dopaminergic neuronal loss observed in PD. This notion led to postulate that stimulation of mitophagy in dopaminergic neurons could serve as a therapeutic target to slow disease progression in PD. However, other reports show that PINK1 loss of function mutation, can also induce the opposite effect, induction of mitophagy [63]. On the other hand, mutations in LRRK2, an autosomal dominant gene involved in PD, have been shown to induce or inhibit autophagy depending on the cell type [33, 64].

6. Concluding Remarks

We have reviewed some of the mechanisms underlying gene mutations associated with autophagy in PD familial cases. Autophagy is a natural cell process to remove protein aggregates, dysfunctional mitochondria, and other potentially toxic proteins or organelles. Whether protein aggregates observed in neurodegenerative disorders are causal factors in the onset of disease is still an open debate. Consistent with this controversy, both deficits and stimulation of autophagy have been reported to underlie neurodegeneration. Thus, current scientific evidence shows that altered protein and organelle clearance, either by excess or deficit, are involved in the onset of PD. However, the mechanisms that could explain this apparently paradoxical behavior are not clear, and further investigation is required in order to use the autophagy machinery and mitochondria and protein-aggregates removal as an effective and safe therapeutic strategy in the treatment of familial and sporadic PD.

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Research Article

N-Acetyl Cysteine Protects against Methamphetamine-Induced Dopaminergic Neurodegeneration via Modulation of Redox Status and Autophagy in Dopaminergic Cells

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Methamphetamine- (MA-) induced neurotoxicity is associated with mitochondrial dysfunction and enhanced oxidative stress. Our previous study demonstrated that MA induces autophagy in a dopaminergic neuronal cell model (N27 cells). The cellular mechanisms underlying MA-induced autophagy and apoptosis remain poorly characterized. In the present study we sought to investigate the importance of GSH redox status in MA-induced neurotoxicity using a thiol antioxidant, N-acetylcysteine (NAC). Morphological and biochemical analysis revealed that MA-induced autophagy in N27 dopaminergic cells was associated with pronounced depletion of GSH levels. Moreover, pretreatment with NAC reduced MA-induced GSH depletion and autophagy, while depletion of GSH using L-buthionine sulfoximine (L-BSO) enhanced autophagy. Furthermore, treatment with NAC significantly attenuated MA-induced apoptotic cell death as well as oxidative stress markers, namely, 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE). Together, these results suggest that NAC exhibits significant protective effects against MA-induced dopaminergic cell death, presumably via modulation of the GSH level and autophagy. Collectively, our data provide mechanistic insights into the role of cellular GSH redox status in MA-induced autophagy and apoptotic cell death, and additional studies are needed to determine the therapeutic effectiveness of cellular redox modifiers in attenuating dopaminergic neurodegeneration *in vivo*.

1. Introduction

Methamphetamine (MA) is a highly addictive psychostimulant that has been shown to cause potent central nervous system stimulant effects. Abuse of this psychostimulant has become an international public health problem, with an estimated 15-16 million users worldwide [1]. MA-induced euphoric effects are accompanied by decreased appetite, hypothermia, paranoia, aggression, and a heightened sense of pleasure [2]. MA neurotoxicity is characterized by long-term reductions in dopaminergic and serotonergic functions, including depletion of dopamine transporter (DAT), serotonin transporter (SERT), serotonin (5-HT), and dopamine (DA) [1]. Magnetic resonance imaging of the brain has shown that chronic use of MA causes neuronal damage [3]. MA abuse has also been linked to increased risk of developing Parkinson's disease (PD). Despite the extensive evidence that substituted amphetamines are neurotoxic, the exact mechanism of action remains poorly understood. A growing body of evidence suggests that MA-induced neurotoxicity involves reactive oxygen species (ROS) and reactive nitrogen species (RNS) [4] and activation of downstream oxidative stress mechanisms. MA enters dopaminergic neurons via dopamine transporter (DAT) and displaces vesicular dopamine. The displaced amines can be oxidized enzymatically and nonenzymatically to form

highly reactive dopamine quinones and reactive oxygen species, leading to enhancement of oxidative stress [5]. In recent years mitochondrial dysfunction has been implicated in the mechanism of MA-induced neurodegeneration [6]. Indeed, exposure to MA decreased mitochondrial membrane potential, increased mitochondrial mass, enhanced protein nitrosylation, and decreased levels of Complexes I, III, and IV of the electron transport chain in primary human cells. Also, antioxidants were found to mitigate the neuronal damage, further suggesting a crosstalk between mitochondrial damage and cellular oxidative stress in MA-induced neurotoxicity [7]. Furthermore, oxidative stress has been observed both *in vitro* and *in vivo*, following MA administration [6, 8, 9].

4-Hydroxy-2-nonenal (4-HNE) is a major oxidative product derived from the breakdown of polyunsaturated fatty acids and related esters [10]. In addition, 4-HNE has been shown to have physiological roles in cell proliferation and differentiation [11] and to cause cellular damage by modification of intracellular proteins [12]. Also, treatment of purified proteins with HNE leads to enzyme inactivation and protein cross linking [13]. Intracellular 4-HNE reacts rapidly with cysteine, lysine, and histidine residues of proteins [14, 15] to form protein adducts. The increases in protein nitration are due to increase in ROS/RNS levels, and 3-nitrotyrosine (3-NT) has served as a marker for the production of reactive nitrogen-centered oxidants (ONOO-, NO_2, etc.). Nitration of active site tyrosine residues has been shown to alter protein structure and function [16, 17]. Under pathological conditions, 3-NT has been suggested to modify both translational and posttranslational processes [18–20]. Therefore, detection of these two biomarkers (4-HNE and 3-NT) following MA treatment would provide strong evidence for the actual presence of oxidative/nitrative damage.

The brain is especially susceptible to oxidative stress due to its capacity to generate large amounts of reactive oxygen species. Glutathione (GSH), a tripeptide comprised of glutamate, cysteine, and glycine, plays essential roles as antioxidant, enzyme cofactor, cysteine storage, major redox buffer, and neuromodulator in the central nervous system [21]. GSH deficiency has been implicated in neurodegenerative diseases including PD. The earliest events causing neurodegeneration include oxidative stress and mitochondrial dysfunction [22]. Oxidative stress during early PD is associated with dramatic reductions in the cellular antioxidant GSH in the SN. GSH depletion precedes both mitochondrial dysfunction and dopamine depletion and is therefore considered the earliest marker of neurodegeneration [23, 24]. In cell culture models, GSH depletion was associated with increased oxidative stress and decreased mitochondrial function [23, 25]. These results suggest that early loss of GSH in the SN of PD patients could be linked to mitochondrial dysfunction and eventually lead to neurodegeneration. Thus, identification of agents that restore intracellular GSH, which might prevent dopaminergic degeneration in PD, is an important endeavor.

N-acetylcysteine (NAC) is an antioxidant and free radical scavenger that increases intracellular GSH at the cellular level. NAC can act as a precursor for GSH biosynthesis as well as stimulator of the cytosolic enzymes involved in glutathione regeneration [26]. NAC has been shown to protect against 4-HNE-induced neuronal death in cultured granule neurons [27]. Based on NAC's beneficial effects, we hypothesize that NAC may also elicit a protective effect against MA-induced neurotoxicity by modulating oxidative damage. Using an *in vitro* model of MA-induced apoptosis, we investigated the mechanisms of neuroprotection exerted by NAC. Our results revealed that NAC replenishes MA-induced GSH depletion and oxidative and nitrative damage. Most importantly, a partial reduction in LC3-II (marker of autophagy) levels was evidenced in MA/NAC-treated cells, thus highlighting the critical role of oxidative stress mechanisms in MA-induced neurotoxicity and autophagy. Thus, our results demonstrate that alteration of cellular redox status serves as a key trigger not only for the induction of apoptosis but also for autophagy.

2. Materials and Methods

2.1. Reagents. (+)-Methamphetamine (MA) was kindly provided by NIDA (National Institute of Drug Abuse, Bethesda, MD). Monochlorobimane (via Fluka Analytical), glutathione S-transferase, 3,5-di-tert-butyl-4-hydroxytoulene (via SUPELCO Analytical), dansylcadaverine, buthionine sulfoximine, and antibodies against β-actin were purchased from Sigma Chemical Company (St. Louis, MO). N-Acetyl-L-cysteine was purchased from Calbiochem (via EMD Biosciences, Gibbstown, NJ). Antibodies against LC3, 3-nitrotyrosine and 4-hydroxynonenal were obtained from Abcam, Inc., Cambridge, MA. Cell Death Detection ELISA PLUS Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

2.2. Cell Culture. The immortalized rat mesencephalic dopaminergic cells (N27 cells) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units penicillin, and 50 μg/mL streptomycin, referred to as complete RPMI medium hereafter. Cells were grown in a humid atmosphere of 5% CO_2 at 37°C until they were 70–80% confluent.

2.3. Treatment Paradigm. Confluent cells were harvested and seeded in the density of 0.2–4 $\times$ 10^6/mL. Cells were pretreated with N-acetyl-L-cysteine (NAC) for 1 hour or buthionine sulfoximine (BSO) for 24 hours prior to the treatment with MA for various time points. Treatments were made in a complete RPMI medium.

2.4. Determination of Cellular GSH. The monochlorobimane fluorometric method was used to determine the cellular GSH levels. Briefly, treated cells were collected, washed with PBS, and sonicated in a lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA and 0.001% 3,5-di-tert-butyl-4-hydroxytoulene (BHT)). Samples were centrifuged at 14,000 g for 10 minutes at 4°C. 1 mM of monochlorobimane and 10 U/mL of glutathione S-transferase in 50 mM Tris, pH 7.4, were dissolved and added to the resulting supernatant. 200 μL

of the mixture was transferred to a black 96-well plate and incubated at 24°C for 30 minutes. The fluorescence of samples was measured using a SPECTRAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA) with excitation at 485 nm and emission at 645 nm.

2.5. Dansylcadaverine Assay. The monodansylcadaverine (MDC) assay to label autophagosomes has been described previously [28]. After treatments, cells were incubated with 0.05 mM MDC in a serum free RPMI medium at 37°C for 30 minutes. Later, cells were harvested and lysed in 10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100. Accumulation of MDC in autophagy vacuoles was measured using a SPECTRAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA) with excitation at 365 nm and emission at 525 nm. The number of cells present in each well was normalized by addition of 0.2 μM ethidium bromide, and the DNA fluorescence was measured with excitation at 530 nm and emission 590 nm. Incorporation of MDC was expressed as specific activity.

2.6. Transmission Electron Microscopy. Cells were grown on coverslips and fixed with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1 M sodium cacodylate buffer, pH 7.2, for 48 hours at 4°C. Samples were washed in PBS and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The samples were then dehydrated in a series of graded ethanol, cleared with ultrapure acetone, and embedded using a modified EPON epoxy resin (Embed 812; Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 70°C. Thick and ultrathin sections were generated using a Leica UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Ultrathin sections were collected onto copper grids and images were captured using a JEM 2100 200 kV scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

2.7. Western Blotting. Treated cells were harvested, washed with 1X PBS (pH 7.4), and lysed in RIPA buffer (Sigma) on ice. Samples were sonicated for 15 seconds on ice and centrifuged at 14,000 × g for 20 minutes at 4°C. Supernatants were collected from each sample and separated on 10–15% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes by electroblotting for 90 mins at 4°C under 100 V. Membranes were blocked for an hour and incubated with rabbit polyclonal to LC3B (1 : 4000), mouse monoclonal 3-nitrotyrosine (3-NT) (1 : 1000), and goat polyclonal to 4-hydroxynonenal (4-HNE) (1 : 500) as primary antibodies for overnight at 4°C. For equal protein detection, mouse monoclonal β-actin (1 : 5000) was used. Later, the membranes were washed several times and incubated with IR Dye 800-conjugated antirabbit IgG (1 : 5000) or Alexa Fluor 680-conjugated anti-mouse IgG (1 : 10000; Molecular Probes, Invitrogen) as secondary antibodies for an hour at room temperature. Membranes were scanned using the Odyssey IR Imaging system (LICOR) and images were analyzed with Odyssey 2.0 software.

2.8. DNA Fragmentation Assay. Measurement of DNA fragmentation was performed using the Cell Death Detection ELISA PLUS Assay Kit [29]. The procedure was similar to the procedure described in our recent publication [30]. Briefly, cells were resuspended in the lysis buffer and incubated for 30 minutes at room temperature. Lysates were centrifuged at 200 × g for 10 minutes. 20 μL of the supernatant was carefully transferred into the streptavidin-coated microplate and incubated for 2 hours in a mixture of HRP-conjugated antibody cocktail that recognizes the nucleosomes in the sample. After thorough washing of the unbound components, an HRP substrate, ABTS, was added into wells. The final reaction product was measured using a spectrophotometer at 405 nm along with 490 nm as the reference reading.

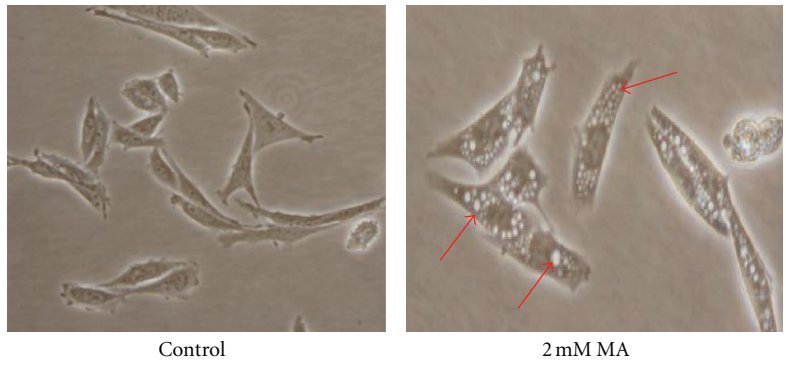
2.9. Data Analysis. Results are presented (PRISM software, GraphPad, San Diego, CA) as fold induction, as compared with the untreated group. Results represent mean ± S.E.M. Statistical analysis was performed by using one-way ANOVA followed by Student Newman–Keuls post-hoc test (PRISM software) in order to compare between groups. P values < 0.05 were considered significant.

3. Results

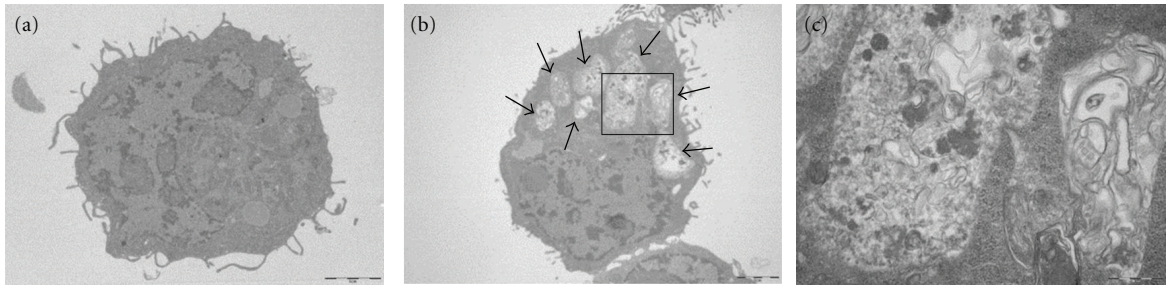
3.1. MA Induces Autophagy. First, we characterized the effect of MA on morphological changes in our mesencephalic dopaminergic neuronal models. As shown in Figure 1(A), 2 mM MA dramatically increased the formation of cytoplasmic vacuoles in N27 dopaminergic cells. The hallmarks of autophagy include the presence of autophagosomes, characterized by double membrane bound vacuoles that contain cytoplasmic material and/or organelles. To examine whether the cell vacuolation induced by MA is related to induction of autophagy, N27 dopaminergic cells were processed after exposure to MA (2 mM) for 12 hours and then ultrastructural analysis was performed using electron microscopy. As shown in Figure 1(B), numerous autophagosomes containing cytoplasmic material and/or organelles were observed in the N27 cells treated with MA (Figure 1(B), b-c).

LC3, an autophagy marker protein, is the mammalian homolog of the yeast ATG8 protein. Upon induction of autophagy, ATG8 protein is covalently modified and redistributed to autophagic vacuoles. In particular, the covalent modification is detected by SDS-PAGE analysis, whereby a shift from LC3-I to LC3-II is evidenced in cells undergoing autophagy [31]. An increase in LC3-II levels was observed starting at 3 hours and reaching a peak at 12 hours following MA treatment (Figure 1(C)). In agreement with electron microscopy analysis, MA treatment increased the expression of LC3-II in a time-dependent manner, thus confirming the formation of autophagosomes during MA neurotoxic insult in dopaminergic neuronal cells.

3.2. MA-Induced Suppression of GSH Levels in N27 Cells Is Attenuated by N-Acetylcysteine (NAC). Reduced glutathione



(A)



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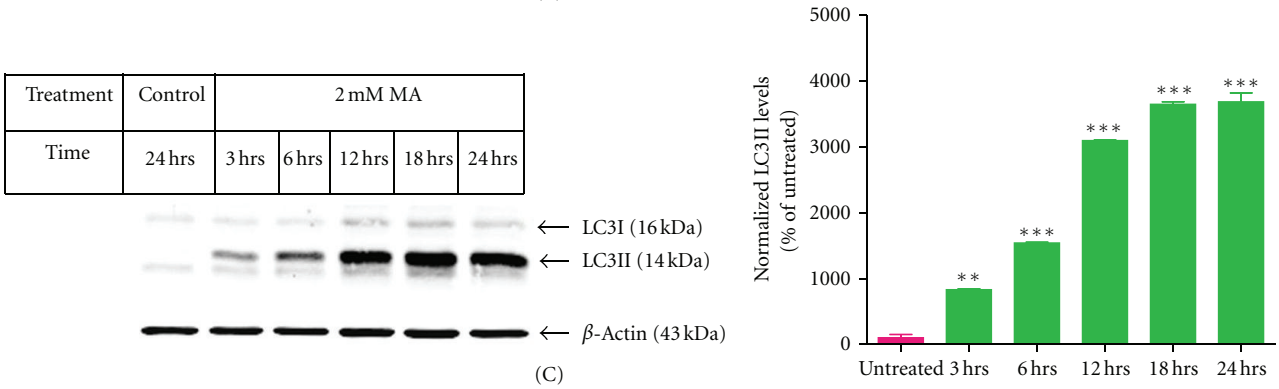
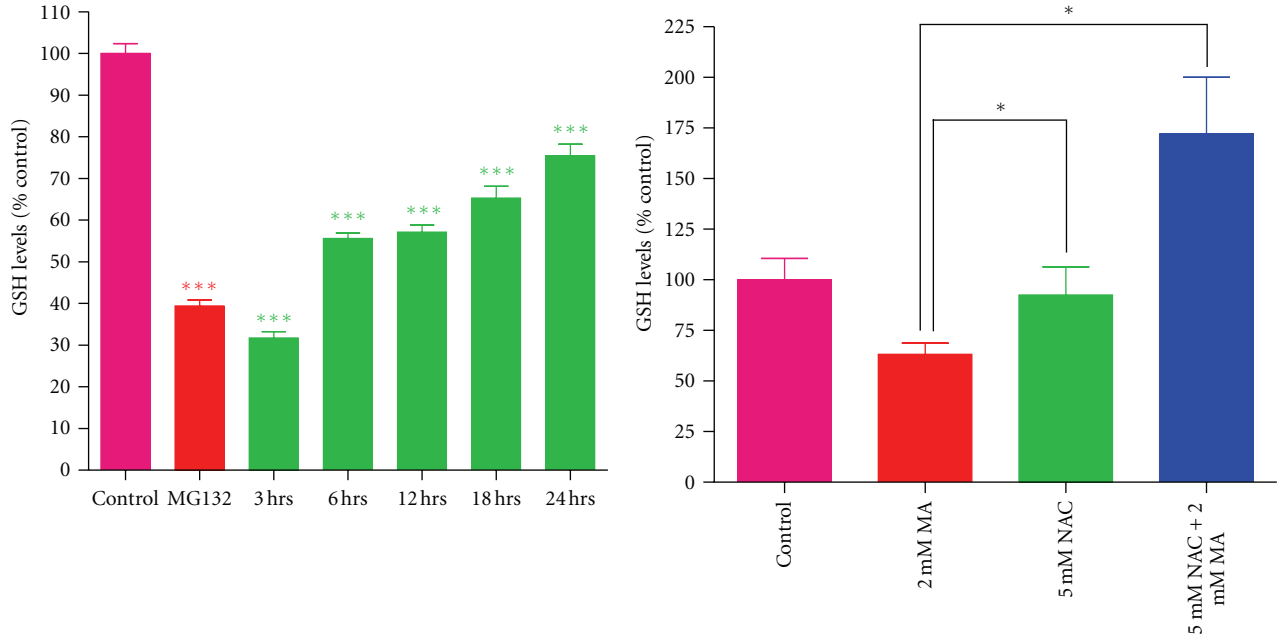


(C)

Figure 1: MA induces autophagy. (A) Representative phase contrast microscopy pictures showing abundant cytoplasmic vacuoles (arrows) in N27 dopaminergic cells treated with MA (2 mM) for 12 hours. (B) Representative transmission electron microscopy image analysis of N27 dopaminergic cells exposed to MA (2 mM) for 12 hours: (a) untreated N27 dopaminergic cells; (b) boxed area; (c) autophagosomes observed in N27 dopaminergic cells treated with MA (2 mM) (arrows). Morphology of autophagosomes is characterized by the formation of double membrane vacuoles harboring damaged organelles (arrows) and insoluble protein aggregates. (C) Time-dependent increase in LC3-II levels. N27 dopaminergic cells were exposed to MA (2 mM) for 3, 6, 12, 18, and 24 hours. Equal loading of protein in each lane is confirmed by probing the membrane with β-actin antibody. Densitometry analysis of LC3-II induction is represented next to the Western blot image. LC3-II bands were quantified and expressed as percentage of untreated control. Data represent mean ± SEM, $n = 2$. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with untreated group.

protects neurons from oxidative damage induced by superoxide, hydrogen peroxide, and other reactive species. Therefore, we examined whether MA alters the GSH levels in dopaminergic cells. As shown Figure 2(a), intracellular stores of GSH were significantly depleted within 3 to 24 hours of MA treatment. While the treatment with 2 mM MA for 3 hours produced a 70% reduction in GSH levels, the 6, 12, 18, 24-hour treatments induced approximately 42%, 40%, 35% and 25% reductions of GHS levels, respectively (Figure 2(a)). Next, we examined whether the GSH precursor NAC can protect cells from MA-induced GSH depletion. N27 dopaminergic cells were pretreated for 1 hour with N-acetylcysteine (5 mM) and then treated for an additional 18 hour with MA (2 mM). MA-induced depletion of GSH levels in N27 cells was attenuated in the presence of NAC, indicating that MA exposure severely compromises the GSH antioxidant redox system.

3.3. Effect of NAC and BSO on MA-Induced Autophagy. To investigate the relationship between enhanced GSH



(a) MA-induced Time dependent changes in GSH levels

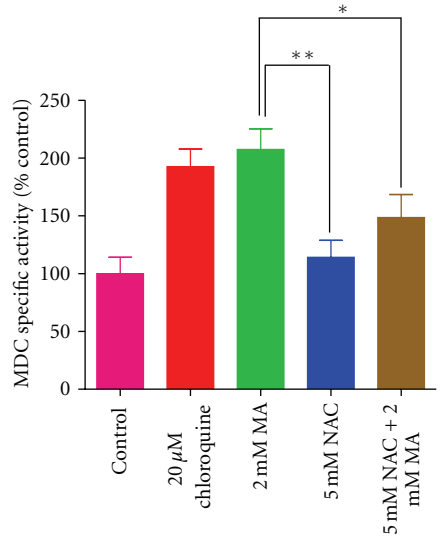
(b) Effect of NAC on MA-induced depletion in GSH levels

Figure 2: Effect of NAC on MA-induced reduction in total GSH levels. (a) Determination of cellular GSH. N27 dopaminergic cells were treated with 2 mM MA for 3, 6, 12, 18, and 24 hours. MA-induced reduction in GSH levels was measured by the monochlorobimane fluorometric method. The data represent mean ± SEM of six individual measurements. Asterisks (***$P < 0.001$) indicate significant differences between MA-treated cells and untreated control cells. Values are expressed as percentage of GSH compared with untreated control. Treatment of N27 dopaminergic cells with 5 μM MG132 is considered as positive control. (b) Determination of cellular GSH levels in N27 dopaminergic cells preincubated (1 hour) with 5 mM NAC prior to MA treatment for 18 hours. The data represent mean ± SEM of four individual measurements. Asterisks (*$P < 0.05$) indicate significant differences between MA-treated cells and NAC alone or NAC with MA-treated cells. Values are expressed as percentage of GSH compared with untreated cells.

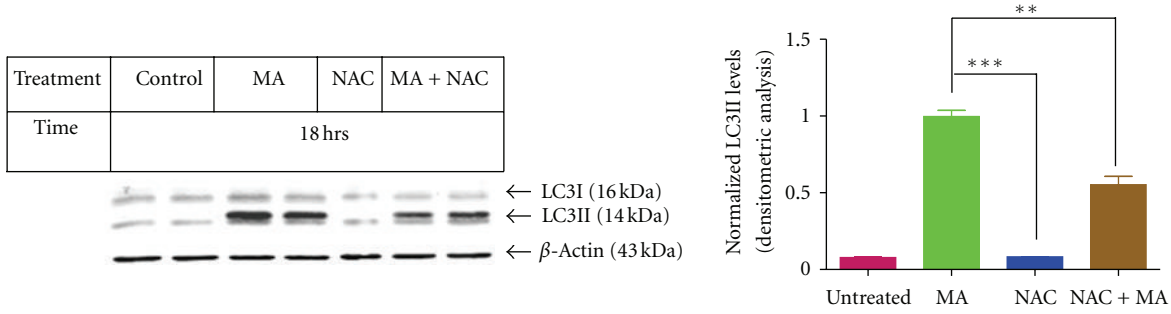
and autophagy, we treated N27 cells with NAC, a GSH precursor, and determined the extent of autophagic vacuole formation by MDC fluorescence assay. Pretreatment with NAC dramatically reduced MA-induced MDC accumulation in autophagic vacuoles by approximately 50% (Figure 3(a)). The major limitation of MDC assay is that it labels acidic compartments comprised of endosomal and lysosomal compartments that have recently fused with autophagic vacuoles, namely, late stage autophagosomes and, therefore, results obtained using MDC as a marker for autophagy should be subject to careful interpretation. For this reason, we performed LC3 Western blot analysis to further clarify the role of NAC in MA-induced autophagy. Figure 3(b) shows NAC (5 mM) treatment partially reversed MA-induced LC3-II expression, further confirming the inhibitory effects of NAC on MA-induced autophagy. In parallel experiments, N27 cells were pretreated for 24 hours with 2 mM L-buthionine-*S*,*R*-sulfoximine (BSO), an inhibitor of GSH biosynthesis, and then were treated for an additional 18 hours with MA (2 mM). MA-induced autophagy was enhanced in the presence of BSO, suggesting that the observed autophagy may be related to the depletion of the endogenous GSH pool.

3.4. Effect of NAC on MA-Induced Increase in Oxidative Stress Markers in N27 Cells. Peroxynitrite nitrates protein-bound tyrosine residues to produce 3-nitrotyrosine (3-NT). Protein-bound 3-NT was determined by Western analysis using an anti-3-NT antibody. Figure 4(a) shows that MA increases the level of protein-bound 3-NT compared with the control group, and NAC has an inhibitory effect on MA-induced upregulation of 3-NT. NAC alone had no effect on protein bound 3-NT levels in N27 cells. MA exposure induced upregulation of 4-hydroxynonenal- (4-HNE-) protein adducts, as revealed by Western blot analysis (Figure 4(b)). Such upregulation was reduced by pretreatment with NAC. NAC alone had no effect on 4-HNE levels in N27 cells.

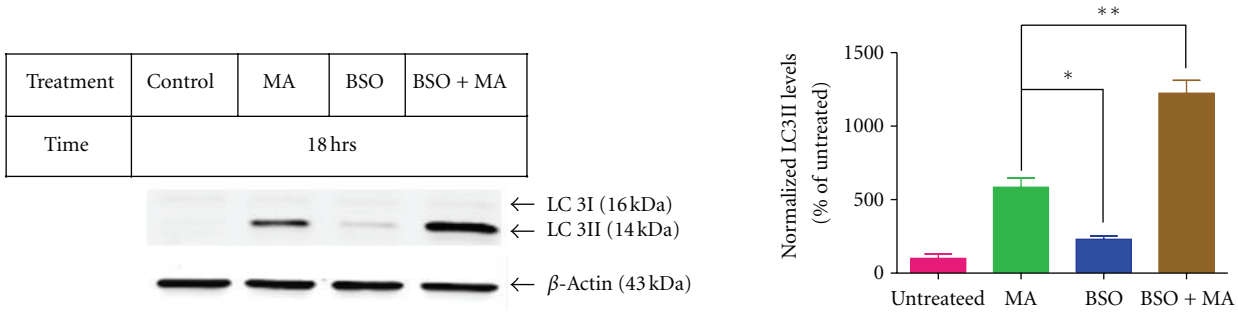
3.5. Effects of NAC on MA-Induced Cell Death. We determined MA-induced neuronal apoptosis by DNA fragmentation enzyme-immunoassay. N27 dopaminergic cells treated with 2 mM MA for 24 hours increased by 2-fold DNA fragmentation, as compared to that of the control (Figure 5). To determine whether NAC suppresses MA-induced apoptosis,



(a) Effect of NAC on MA-induced MDC accumulation

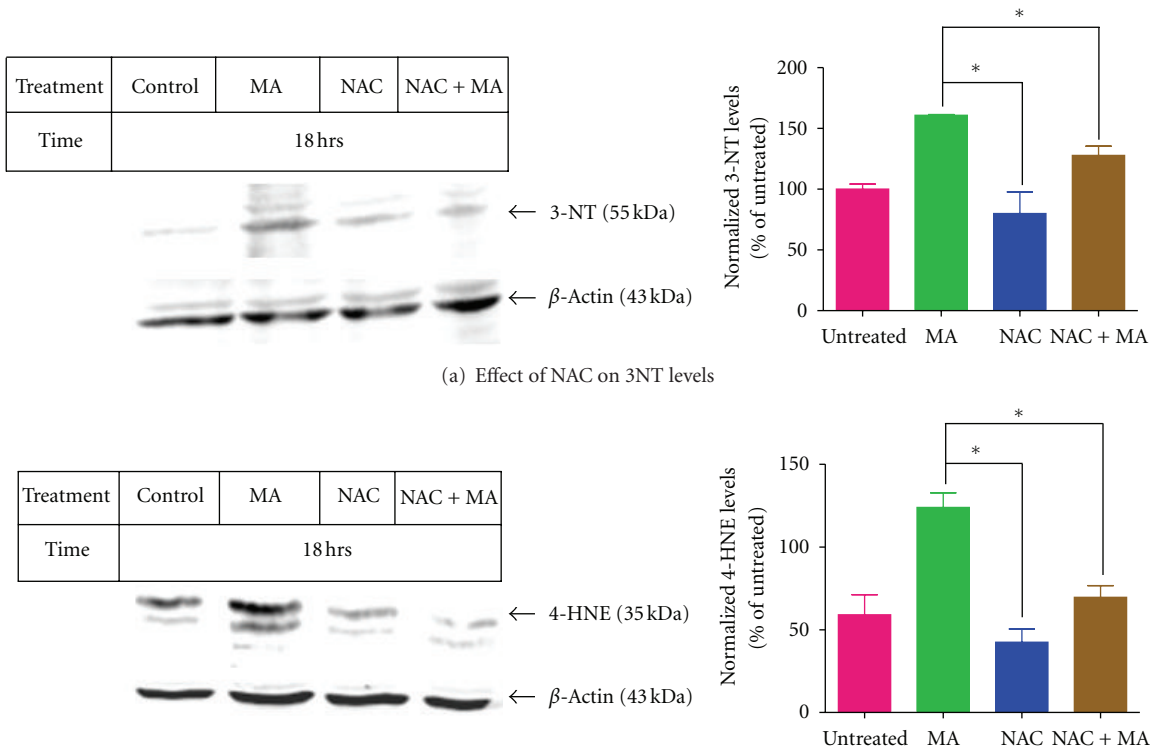


(b) Effect of NAC on MA-induced upregulation of LC3-II



(c) Effect of BSO on MA-induced upregulation of LC3-II

Figure 3: (a) Effect of NAC on MA-induced accumulation of monodansylcadaverine (MDC) in autophagy vacuoles. N27 dopaminergic cells were preincubated with 5 mM NAC for an hour prior to exposure to MA (2 mM) for 18 hours. MA-induced changes in intracellular MDC fluorescence were measured as indicated in Methods. The data represent mean ± SEM of four individual measurements. Asterisks ($^*P < 0.05$ and $^{**}P < 0.01$) indicate significant differences between MA-treated cells and NAC alone or NAC with MA-treated cells. Values are expressed as percentage of MDC specific activity compared to untreated control cells. Treatment of N27 dopaminergic cells with 20 μM chloroquine is considered as test control. (b) NAC reduced LC3-II levels in N27 dopaminergic cells treated with MA. Western blot analysis of LC3-II expression in N27 dopaminergic cells after exposure to MA (2 mM) with or without 5 mM NAC during 18 hour treatment is presented. Equal loading of protein in each lane is confirmed by probing the membrane with β-actin antibody. Densitometry analysis of LC3-II induction ($n = 2$) is represented next to the Western blot image. (c) BSO enhances the expression of LC3-II. N27 dopaminergic cells were pretreated with 100 μM BSO for 24 hours and treated with MA for another 18 hours. Equal loading of protein in each lane is confirmed by probing the membrane with β-actin antibody. Densitometry analysis of LC3-II induction is represented next to the Western blot image. LC3-II bands were quantified and expressed as percentage of untreated control. Data represent ± SEM, $n = 2$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with MA-treated cells.



(a) Effect of NAC on 3NT levels

(b) Effect of NAC on 4HNE levels

Figure 4: NAC attenuates markers of lipid and protein oxidative damage. (a) Western blot analysis of 3-NT detection and (b) Western blot analysis of 4-HNE detection in N27 dopaminergic neurons preincubated with 5 mM NAC and treated with or without MA (2 mM) for 18 hours. Equal loading of protein in each lane is confirmed by probing the membrane with β-actin antibody. Densitometry analysis of 3-NT and 4-HNE induction is represented next to the Western blot image. 3-NT and 4-HNE bands were quantified and expressed as percentage of untreated control. Data represent ± SEM, $n = 2$. $^*P < 0.05$ compared with MA-treated cells.

we treated the cells with NAC for 1 hour prior to MA treatment. While NAC by itself had little or no effect, NAC treatment showed a partial reversal of MA-induced apoptosis ($P < 0.001$). Collectively, these data indicate that NAC can attenuate MA-induced apoptotic death possibly by restoring GSH levels in dopaminergic cells.

4. Discussion and Conclusions

In this study we evaluated the neuroprotective potential of NAC on MA-induced autophagy and apoptosis in the mesencephalic dopaminergic neuronal cell model. We also examined the relationship between cellular redox status, autophagy, and apoptotic cell death following MA exposure. MA produced a substantial reduction in surviving dopaminergic neurons, marked by early depletion of GSH, induction of autophagy, and upregulation of oxidative stress markers, namely, 3-NT and 4-HNE. Indeed, MA-induced oxidative stress has been shown to be a critical event in neurotoxicity. NAC was chosen in this study because of its potent thiol-based antioxidant effect. NAC was able to partially attenuate MA-induced apoptotic cell death, upregulate GSH levels, partially attenuate the autophagy marker LC3-II, and completely abrogate oxidative stress markers. There are several possible mechanisms by which NAC might prevent dopaminergic neuronal cell death. For example, NAC might prevent neuronal cell death via its antioxidant effects capable of reducing reactive oxygen species (ROS). Alternatively, NAC could also enhance intracellular levels of GSH and serve as a reducing agent. Nevertheless, our study highlights the central role of cellular redox status both in the mechanism of neuroprotection and modulation of autophagy. Previous studies have shown that NAC suppresses MA-induced neurotoxicity in striatal neurons [32] and in immortalized human brain endothelial cells [33], but the mechanisms associated with the protective effect were not explored. Our results suggest that NAC treatment restores MA-induced imbalance in cellular redox status and thereby prevents the neuronal cell death.

The cellular mechanism underlying the proapoptotic effects of MA in dopaminergic neurons remains poorly understood. Multiple mechanisms, including mitochondrial dysfunction, oxidative stress, and apoptosis, have been implicated in MA-induced neurotoxicity [1]. The involvement of oxidative stress in MA-induced neurotoxicity has been studied extensively, whereby accumulation of oxidatively damaged lipids, proteins, and DNA has been shown to occur in the brain regions of animal models as well as in *in vitro* cell culture models of neurodegeneration [34–36]. In fact, oxidative stress has been identified as an early event in

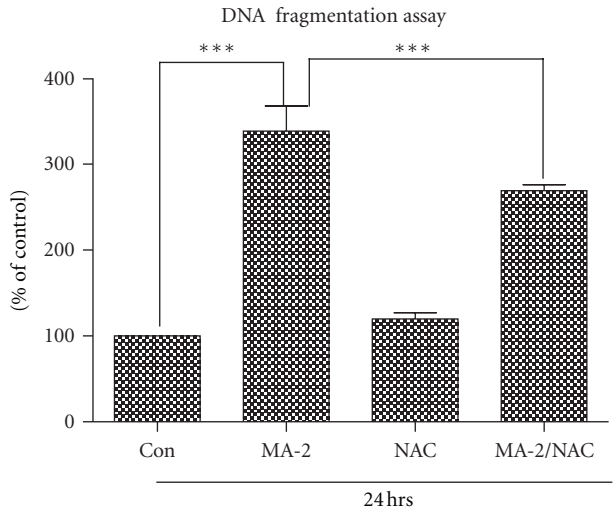


Figure 5: Effect of NAC on MA-induced apoptotic cell death in N27 cells. Cells were pretreated with 5 mM NAC for 1 hour followed by treatment with MA (2 mM) or PBS for 24 hours. DNA fragmentation was quantified using a cell death detection using Roche Elisa PLUS kit. The data are expressed as percentage of DNA fragmentation compared with untreated control cells, and asterisks (***$P < 0.01$) indicate significant differences between untreated control group with MA-treated group and MA-treated group with NAC group with MA treatment.

dopaminergic degeneration because neurotoxicity has been shown to be attenuated by antioxidants such as Trolox and GSH [37]. Additionally, GSH depletion has also been shown to result in the loss of protein sulfhydryls, including transporter proteins [38]. In line with these findings, we observed that MA-induced apoptotic cell death was preceded by early and pronounced depletion of GSH. Pretreatment of MA-treated cells with NAC restored the GSH levels and decreased apoptotic cell death, indicating that NAC had replenished the GSH levels in these cells, thereby attenuating MA-induced oxidative neuronal cell death. At the cellular level, assessment of reduced GSH is considered to be a marker of cellular antioxidant defense, and a reduction in the levels of GSH is an indicator of oxidative stress [39]. A reduction in GSH alone can act as an inducer of apoptotic events. For example, in a previous report BSO-induced intracellular GSH depletion was found to induce ROS generation and PKCδ activation, thereby resulting in cell death in neuroblastoma cells [40]. Also, GSH depletion in a B cell lymphoma cell has been shown to induce ROS-mediated apoptosis [41]. Thus, our study raises the possibility that oxidative stress-dependent GSH depletion may play a role in MA-induced neurotoxicity. The exact nature of MA's effect on endogenous GSH levels remains controversial. For example, MA was found to increase hippocampal, frontocortical, and striatal levels of GSH in both rats and mice [42] following a short treatment with MA; however, other studies found a reduction in striatal GSH after MA administration [43–45]. In a similar fashion, in postmortem brains of MA abusers, a dramatic loss of DA in the caudate was accompanied by a decrease in GSH and increase in GSSG, the oxidized form of GSH [46]. Increasing evidence shows that administration of MA causes a prominent oxidative stress response, which, in turn, leads to severe nigrostriatal dopaminergic neurotoxicity, as evidenced by loss of striatal dopamine transporter (DAT) [1]. In this context, ROS-dependent oxidative stress mechanisms have been suggested in animals that were administered MA [47–51]. Several *in vivo* studies showed the involvement of neuronal nitric oxide synthase (nNOS) in MA-induced neurotoxicity. For example, administration of MA to mice deficient in nNOS or treatment with nNOS pharmacological inhibitors was found to significantly attenuate MA-induced striatal DA and DAT depletion [47, 49]. Other studies demonstrated the overexpression of NOS in the MA-treated mouse striatum [51]. Since both ROS and RNS have very short half-lives, a reliable approach to demonstrate the interaction between nitric oxide and superoxide is the formation of peroxynitrite, which can be determined by measuring the levels of 3-NT residues. In our studies, increased levels of 3-NT following MA suggest that cellular dysfunction is related to excessive production of peroxynitrite. Also, increased production of 3-NT levels during MA treatment positively correlated with cell death. A role for peroxynitrite in MA-induced neurotoxicity has been documented using selenium (a scavenger of two-electron oxidants), which demonstrates a neuroprotective effect in MA-induced neurotoxicity [52]. Furthermore, peroxynitrite has been shown to inhibit DAT and, therefore, inhibitory effects on DAT would favor cytosolic DA accumulation, which would lead to increased generation of ROS within dopaminergic neurons. It is also probable that early depletion of GSH might induce nitrosative damage to mitochondrial proteins, leading to activation of mitochondria-mediated cell death signaling events. Indeed, possible involvement of protein nitration of complex-1 inhibition by peroxynitrite in GSH depleted cells has been reported [53]. Also, NO might be the primary agent involved in mitochondrial dysfunction following acute GSH depletion in dopaminergic cells [54].

Another marker of increased oxidative stress is lipid peroxidation [9]. MA treatment resulted in increased levels of 4-HNE after 18 hours of MA treatment. Lipid peroxidation has been shown to persist for up to 24 hours after MA administration in rodents [55–57]. Also, GSH conjugates may combine with NO to form nitrosoglutathione and also with lipid peroxidation adducts 4-HNE [58, 59]. Alternatively, peroxynitrite is a potent oxidant species that has been found to cause lipid peroxidation independently [58, 60]. In the present study, treatment with NAC significantly reduced the levels of 4-HNE and apoptosis, indicating the importance of oxidative stress mechanisms in MA-induced cell death. In fact, in a recent study [57] MA was found to cause lipid peroxidation-mediated damage to Parkin and 26 S proteasome, thereby resulting in early loss of ubiquitin proteasomal (UPS) function [57]. Recently, we demonstrated that MA treatment impairs UPS function and triggers autophagy in both cell culture and animal models [61]. Furthermore, we demonstrated that genetic ablation or siRNA-mediated gene silencing of redox sensitive kinase, protein kinase c delta (PKCδ), conferred resistance

against MA-induced dopaminergic apoptotic cell death in N27 cells, suggesting a causal role for PKCδ in MA-induced dopaminergic neurodegeneration. Additional studies from our laboratory also demonstrated that ROS is an integral component of the activation of a redox sensitive kinase PKCδ because superoxide scavenger MnTBAP attenuated Parkinsonian toxicant MPP-induced proteolytic activation of kinase and cell death [62] while prooxidants hydrogen peroxide [63] and 6-hydroxydopamine induced apoptosis through PKCδ activation in N27 dopaminergic cells [61]. Taken together, amelioration of MA-induced oxidative insult by NAC may be related to dampening of PKCδ proteolytic activation and associated apoptotic signaling events. Studies have demonstrated that acute administration of MA results in increased aldehyde accumulation in animal models of MA-induced neurodegeneration [9, 42, 64]. MA-induced oxidative stress is functionally linked to mitochondria-dependent apoptosis. Mitochondria serve as indispensable power houses of the cell and consume large amounts of oxygen in the mitochondrial respiratory chain pathway, resulting in production of a major source of ROS generation. Furthermore, a recent study [65] showed that autophagy is induced through oxidative inactivation of Atg4. Our results with MA-induced 3-NT and 4-HNE levels suggest that generation of nitrosylated oxidative species and lipid peroxides is presumably linked to activation of PKC delta-dependent mitochondria-mediated apoptotic cell death events, which may be central to MA-induced dopaminergic neurotoxicity.

ROS-mediated events may not be the sole redox-related event involved in the regulation of autophagy. Other factors, such as GSH redox status, have also been shown to regulate autophagy [65, 66]. In the present study, we suggest that altered intracellular GSH content can modulate autophagy because (i) MA treatment was associated with an early depletion of GSH content; (ii) the addition of NAC replenished the intracellular level of GSH and partially prevented autophagy; and (iii) the depletion of cellular GSH by BSO increased the levels of autophagy. The fact that NAC pretreatment significantly increased GSH levels illustrates the significance of initial cellular redox state in influencing the cell response to MA exposure and supports the conclusion that observed changes may occur via a shift in intracellular redox state.

In conclusion, the present results reveal that loss of cellular levels of GSH is one of the pivotal mechanisms involved in MA-induced neurotoxicity and autophagy in mesencephalic dopaminergic neuronal cells and that treatment with NAC partially reverses MA-induced apoptotic cell death, possibly by replenishing GSH levels. Our results also indicate that MA-induced neurotoxicity is associated with increased 4-HNE levels and 3-NT adduct formation. Moreover, scavenging of free radicals such as RNS and ROS using NAC also partially attenuated MA-induced upregulation of autophagy. To the best of our knowledge, this is the first report demonstrating that NAC pretreatment can ameliorate MA-induced autophagy, highlighting the importance of redox status of the cell in MA-induced dopaminergic neurodegeneration. Further studies will be necessary to confirm the effect of NAC on redox status and autophagy, and the relevance to MA-induced dopaminergic degeneration in animal models.

Acknowledgments

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Review Article

Parkinson's Disease: Leucine-Rich Repeat Kinase 2 and Autophagy, Intimate Enemies

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Parkinson's disease is the second common neurodegenerative disorder, after Alzheimer's disease. It is a clinical syndrome characterized by loss of dopamine-generating cells in the substancia nigra, a region of the midbrain. The etiology of Parkinson's disease has long been through to involve both genetic and environmental factors. Mutations in the leucine-rich repeat kinase 2 gene cause late-onset Parkinson's disease with a clinical appearance indistinguishable from Parkinson's disease idiopathic. Autophagy is an intracellular catabolic mechanism whereby a cell recycles or degrades damage proteins and cytoplasmic organelles. This degradative process has been associated with cellular dysfunction in neurodegenerative processes including Parkinson's disease. We discuss the role of leucine-rich repeat kinase 2 in autophagy, and how the deregulations of this degradative mechanism in cells can be implicated in the Parkinson's disease etiology.

1. Parkinson's Disease

The ability to control body movement is an inherent human capacity. It is difficult to imagine the normal performance of many daily and routine activities without a normal control of movement. Nevertheless, many people experience body movement disorders and struggle daily with their handicap. Since antiquity, there have been a multitude of references to individuals with movement disorders. Galen and Hippocrates described people who presented classic symptoms of Parkinson's in ancient Greece. References to the disease also occur in the papyrus writings of the Egyptians of the 19th dynasty and the classic Chinese texts of the 1st century BC.

However, it was not until 1817 that James Parkinson (1755–1824), a British physician with ample clinical experience, published *"An Essay on the Shaking Palsy."* PD is the second common neurodegenerative disorder, after Alzheimer's disease. Estimated prevalence rate is about 300/100,000 population and incidence and prevalence rates rise with advancing age [1]. Initial symptoms, which typically begin at or around age 60, reaching an important disability within 5 or 15 years later [2]. The origin of the disorder lies in the loss of at least 50% of the neurons in an area of the mesencephalon known as the substantia nigra pars compact. These neurons show a characteristic dark pigmentation because of the presence of melanin. Under normal physiological conditions, these neurons produce dopamine, which provides inhibitory signals to the corpus striatum to control the execution of smooth and precise movements. In a person with Parkinson's, the death of neurons in the substantia nigra leads to a depletion of dopamine in the corpus striatum [3], which is responsible for the patients' motor symptoms, especially akinesia [4].

Over time, PD has been suggested to have a multifactorial etiology, in which both genetic and environmental factors are included [5]. In 1988, Gowers introduced the possibility of a hereditary basis for PD, given the family history

of a considerable number of patients with the disease. Therefore, knowledge about the genetic factors involved in the disease is essential when clarifying the possible causes and mechanisms underlying its development. Epidemiological studies have revealed that most cases of individuals with the illness are sporadic and that only 5–10% shows a pattern of hereditary transmission, which highlights the importance of environmental factors in the origin of the illness. As a result, it is postulated that the cause of the disease can be attributed to an interaction between hereditary and environmental factors, where the genetic factor predisposes but does not determine the development of the illness. A family history of PD constitutes a risk factor at the time of PD development [6]. Family cases of Parkinsonism were observed, which led to an increase in studies evaluating a possible genetic predisposition to developing PD. In 1997, an autosomal dominant mutation of the *PARK1* gene that coded for the α-synuclein protein was identified in Italian and Greek families who suffered from a hereditary form of PD [7]. This finding, along with the discovery of α-synuclein as the major component of Lewy bodies [8], led to greater interest in the genetic aspects of PD. In the following years, other genes implicated in PD were discovered (Table 1). In 1998, the *PARK2* gene, which codes for the parkin protein [9], was identified; it was found to be mutated in an inherited juvenile variation of PD. Subsequent studies identified new key mutations in PD, such as the mutation of the DJ-1 protein in Dutch and Italian families [10], which is responsible for an autosomal recessive variation of PD. A mutation in the *PARK6* gene coding for the PINK1 protein has been described; the mutation could originate from a metabolic error and neuronal death in the substantia nigra [11]. In recent years, the number of studies related to the *PARK8* gene, which codes for the leucine-rich repeat kinase 2 (LRRK2) protein and could be directly associated with the development of PD, has risen dramatically.

2. Leucine-Rich Repeat Kinase 2

In 2004, mutations in the *PARK8* gene were described as one of the major genetic causes associated with hereditary Parkinsonism [12]. The *PARK8* gene was studied for the first time in the Japanese Sagamihara family; members who suffered from PD responded positively to treatment with L-Dopa and had idiopathic Parkinsonism disease characteristics [13]. This protein was later associated with PD by studies in two other families (German and Canadian) who also presented late-onset hereditary autosomal dominant Parkinsonism [14].

The *PARK8* gene is located on the 12q12 chromosome and has 51 exons that code for a 2527 amino acid protein with molecular weight of 285 kDa. This protein has multiple denominations, including PARK8, RIPK7, or ROCO2. However, the most utilized names are *leucine-rich repeat kinase* 2 (LRRK2) because of the presence of a domain rich in leucine, or *dardarin* (from the Basque word *dardara*, which means trembling, one of the most characteristic symptoms of PD).

LRRK2 (Figure 1) is a protein that has a homodimer structure [15], which suggests that it could have the capacity to self-regulate its kinase activity and GTPase activity [16]. Recent studies have indicated that LRRK2 is predominantly found in monomer form and that it only takes a homodimer configuration to regulate enzymatic activity [17]. LRRK2 contains multiple conserved domains including Ankyrin, leucine-rich repeat (LRR), WD40, a MAPKKK kinase, and GTPase.

More than 20 mutations are known in the LRRK2 structure [18] and mutations studied most relevant in the LRRK2 structure, *G2019S*, and *R1441*, are locates the kinase and GTPase domain, respectively. The *G2019S* mutation shows reduced penetrance (as low as 24%), however, *R1441* mutation is highly penetrant (95% at older ages) [19].

Various studies have associated changes in LRRK2 kinase activity with cellular death processes. The kinase domain of LRRK2 is highly homologous with other MAPKKKs of the tyrosine-kinase group [20], in which various mutations have been detected. These mutations have been mostly found in the preserved DF/YG sequence, which has been linked to PD. The G2019S mutation is found in the Mg^{2+} union site of the kinase domain. The exchange of glycine for serine facilitates the access of the kinase domain to its substrates, thereby augmenting its capacity for autophosphorylation 2.5-fold and its capacity to phosphorylate other substrates 3-fold. The I2020T mutation is found in the zone adjacent to the 2019 residue, and it therefore influences the activation site of the kinase domain. The exchange of an isoleucine for a tyrosine next to the DYG activation site increases the autophosphorylation capacity of LRRK2 by 40%. Such a mutation can also modify the specificity for substrates and result in an increase in toxicity [21].

2.1. Functions LRRK2. LRRK2 is expressed in organs within the central nervous system and outside the central nervous system, including the kidneys, lungs, liver, heart, and leukocytes [22]. LRRK2 is expressed in the different areas of the brain, with ample expression in the cortex, the basal ganglia, the cerebellum, and the hippocampus [23]. It is also present in the substantia nigra of the mesencephalon, although at low levels [24]. Thus, LRRK2 is found in areas that contain dopaminergic neurons. The interruption of dopamine transmission does not affect the expression of LRRK2, although it is not known how this change affects the functionality of the protein. Curiously, an increase in the expression of LRRK2's mRNA has been observed upon stimulation of MPTP [25]. LRRK2 is primarily a cytosolic protein, although 10% of the protein is located in the external membrane of the mitochondria [23]. LRRK2 is also associated with the plasma membrane, the Golgi apparatus, microtubules [26], synaptic vesicles [27], and lipid rafts [28].

Because of the number of domains in its structure, the LRRK2 protein can interact with various other proteins. According to Dächsel et al., 3 groups of proteins can interact with LRRK2: the chaperone-mediated response group, the cytoskeletal interaction group, and the kinase activity proteins [29]. However, previous studies discovered multiple new proteins that also interact with LRRK2, including β-tubulin and actin, which interact with the Roc domain

Table 1: Genes associated with Parkinson's disease linkage.

Gene	Locus	Protein name	Inheritance pattern	Description
PARK 1/4	4q21.3-q22	α-synuclein (SNCA)	AD	Lewy's body component
PARK 2	6q25.2-27	Parkin	AR	E3 ubiquitin-protein ligase
PARK 3	2p13	¿?	AD	¿?
PARK 5	4p14	UCH-L1	AD	Ubiquitin C-terminal hydrolase
PARK 6	1p35-36	PINK1	AR	Mitochondrial kinase
PARK 7	1p36	DJ-1	AR	Chaperone mitochondrial kinase
PARK 8	12q12	LRRK2	AD	Kinase/GTPase
PARK 9	1p36	ATP13A2	AR	Cationic transport
PARK 10	1p32	¿?	AD	¿?
PARK 11	2q36-q37	GIGYF2	AD	Receptor tyrosine phosphorylation regulation
PARK 12	Xq21-q25	¿?	X-linked	¿?
PARK 13	2p13	HTRA2/OMI	AD	Serine protease
PARK 14	22q13.1	PLA2G6	AR	Phospholipase A2
PARK 15	22q11.2	FBXO7	AR	E3 ubiquitin-protein
PARK 16	1q32	¿?	¿?	ligase¿?

AD: autosomal dominant; AR: autosomal recessive.

of LRRK2 independently of GTP, and are considered kinase substrates of LRRK2 [30]. As such, LRRK2 could be implicated in the reorganization processes of the cytoskeleton [31].

When we inhibit the interaction between LRRK2 and Hsp90 (heat shock protein 90), which is responsible for the regulation of the folding of other proteins, the degradation of Hsp90 is mediated by proteasomes. Therefore, Hsp90 could be responsible for maintaining the stability of LRRK2. Following an alteration of this stability, the elimination of LRRK2 occurs. In the case of mutations that compromise cellular viability, this destabilization could be utilized to degrade the molecule that is causing the cellular damage, as is the case with the G2019S mutation of LRRK2 [32]. CHIP (Hsp70-interaction protein) is another protein that has been studied for its interaction with LRRK2 [33] and that could affect the molecular stability of LRRK2. Similar interaction exist with the 14.3.3 proteins that are directly implicated in the maintenance of the stability of LRRK2 [34], which is dependent upon the LRRK2's autophosphorylation capacity [35].

LRRK2 can also influence cellular death processes because of its interaction with proteins such as FADD (Fas-associated protein with dead domain), which is implicated in the activation of apoptosis. Recent studies have indicated a relationship between LRRK2 and the activation of programmed cellular death, which suggests that FADD/caspase 8 contributes to the cellular death induced by LRRK2 [36].

Rab5b is implicated in the regulation of endocytosis and interacts with LRRK2. It could play a fundamental role in the synaptic function that modulates the endocytosis of synaptic vesicles [27].

Several studies have associated LRRK2 with other proteins related to PD, such as parkin [37], PINK-1, and DJ-1 [38]. Studies have also related LRRK2 to α-synuclein, indicating that an increase in LRRK2 produces an acceleration of neuropathologies caused by mutations in α-synuclein [39].

The interactions of LRRK2 with MAPKs such as ERK (kinases activated by extracellular signals) [40], JNK (N-terminal of C-Jun kinases), and p38 [41] have also been studied, especially with regard to the transport of proteins through synaptic vesicles [27] and the process of ubiquitination [33]. Some studies have also associated LRRK2 with autophagy [42] and apoptosis [36].

3. Autophagy

The maintenance of the correct balance between the synthesis and degradation of all cellular constituents is vital for the survival of the cell. The cell maintains a continual process of renewing its organelles and proteins, and it is necessary to discard the material that has been synthesized but is no longer useful to the cell. The unneeded material is degraded and reused to obtain energy or synthesize new molecules. The cell has two primary mechanisms for breaking down cellular components: the ubiquitin-proteasome system [43] and autophagy [44].

The term autophagy is derived from two Greek words: "auto," which means self, and "phagia," which indicates the action of eating (autophagy literally means "to eat oneself"). Autophagy is a catabolic intracellular mechanism that has been highly preserved throughout evolution; it is the process by which the cell recycles or degrades proteins or damaged cytoplasmic organelles (Figure 2) [45]. Autophagy was described by Christian de Duve in the 1960s, however, it was not until the 1990s that the genes involved in the process were identified in yeast. Since then, these genes have been termed *Atgs* genes (autophagy-related genes) [46]. Currently, the number of papers published annually on autophagy is exponentially growing because studies are revealing the importance of this mechanism in development and in various illnesses.

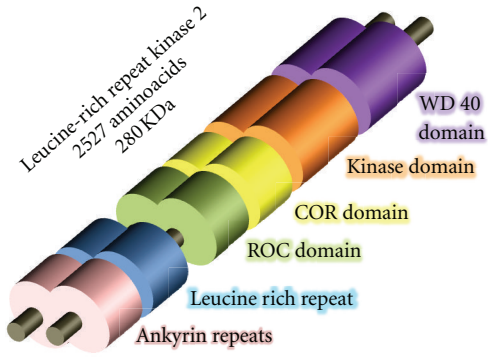

Figure 1: LRRK2 domain structure with homodimeric conformation. LRRK2 is a protein that contains ankyrin repeats, leucine-rich repeats, a catalytic core of the protein contains a GTP-binding ROC (Ras of complex proteins), COR domain (C-terminal of ROC), kinase domain. At the C-terminus is a WD40 repeat followed by a short C-terminal tail.

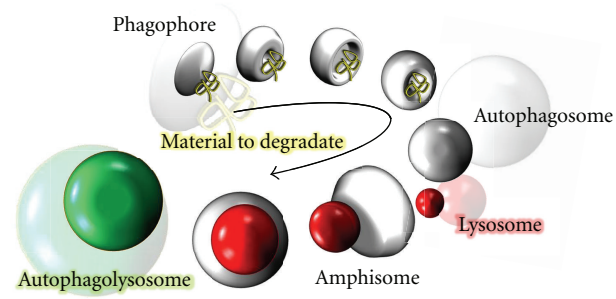

Figure 2: Schematic Illustration on 3D of the autophagy flux. The first step consists of the formation of isolation membranes (phagophore) and elongation of this membrane for sequester the material to degraded (autophagosome). Finally a lysosome is fused with the autophagosome (autophagolysosome) and the cargo is degraded.

An important role of autophagy has been described in neonatal development [47] and in illnesses such as cancer [48], cardiomyopathies [49], musculoskeletal problems, diseases of adipose tissue, and neurodegenerative processes [50, 51]. In fact, it has been described dysfunctional autophagy as one of the failing cellular mechanisms involved in the pathogenesis of idiopathic PD [52]. Studies have also associated autophagy with aging. It has been observed that a hypercaloric diet accelerates the aging process compared with a calorie-restricted diet but not malnourishment. Individuals with a hypocaloric diet had fewer incidences of cancer, cardiovascular disease, and diabetes, and they had a later mortality [53].

Therefore, the importance of the correct regulation of autophagy for maintaining cell viability is clear. However, autophagy involves a complex regulation of cellular recycling (Figure 3). Despite the research efforts undertaken in recent years, many gaps remain in the understanding of the exact regulatory mechanism of autophagy.

The existence of various negative regulators of autophagy is known, among which the mTOR (the mammalian target of rapamycin) protein is one of the most studied autophagy repressors. mTOR is a protein kinase that is active under favorable cellular conditions, repressing autophagy through the phosphatase PP2A [54]. The phosphoinositido3-kinase (PI3K) class I route is also implicated in the negative regulation of autophagy through direct interactions with mTOR [55]. Like PI3k class I, NF-κB exercises negative regulation by activating mTOR [56]. Another molecule that negatively regulates autophagy is Bcl-2.I, which can inhibit the activation route via the PI3K class III pathway (through interactions with Beclin-1) and through the protection provided by Bcl-2 to the mitochondrial membrane of the cell [57].

However, many pathways are capable of positively regulating autophagy. The most well-known pathway is the PI3K class III Beclin-1-dependent route, which has been implicated in the activation of the first formation phases of autophagosomes [58]. The stimulation of autophagy by ERK pathway is known [59], and in recent studies, the presence of reactive oxygen species (ROS) has been involved in the regulation of autophagy [60].

4. Autophagy-LRRK2

The role of LRRK2 in such complex regulation is complicated. However, certain information is available that directly implicates it in the regulation of this cellular degradation mechanism. The first indication of this possible interaction was the discovery that an endogenous part of LRRK2 is anchored to membranous structures of the cell, including the ER and endosomes [23], and that the overexpression of the mutant form of *G2019S* of LRRK2 in neuronal cells induces the accumulation of autophagic structures [42], as also observed in nonneuronal cells [61], iPSC-based model [62] or transgenic mice [63]. However, LRRK2 interacts with various proteins that are implicated in the regulation of autophagy, such as CAMKK-β/AMPK, which is dependent on Ca^{2+} and can induce the accumulation of autophagosomes [64]. In *in vivo* studies, a depletion of LRRK2 is related to a decrease in 4EBP, which is the target of mTOR [65]. This finding directly associates the LRRK2 protein with aging and autophagy processes. However, interestingly, has been observed a age-dependent bi-phasic alteration in autophagic activity in LRRK2 knockout accompanied by modulations in levels of lysosomal proteins and proteases at different months of age [66].

From the studies previously indicated, it is obvious that the LRRK2 protein participates in the regulation of the autophagic cellular process, and as changes in protein activity affect the deregulation of autophagy, it becomes harmful for the cell. Nevertheless, the exact mechanism of the regulation is still unknown.

There are different pathways in the regulation of autophagy in which the LRRK2 protein is involved.

4.1. Regulation of Autophagy by Nutrient Deprivation. An equilibrium between the energy available for the cell and

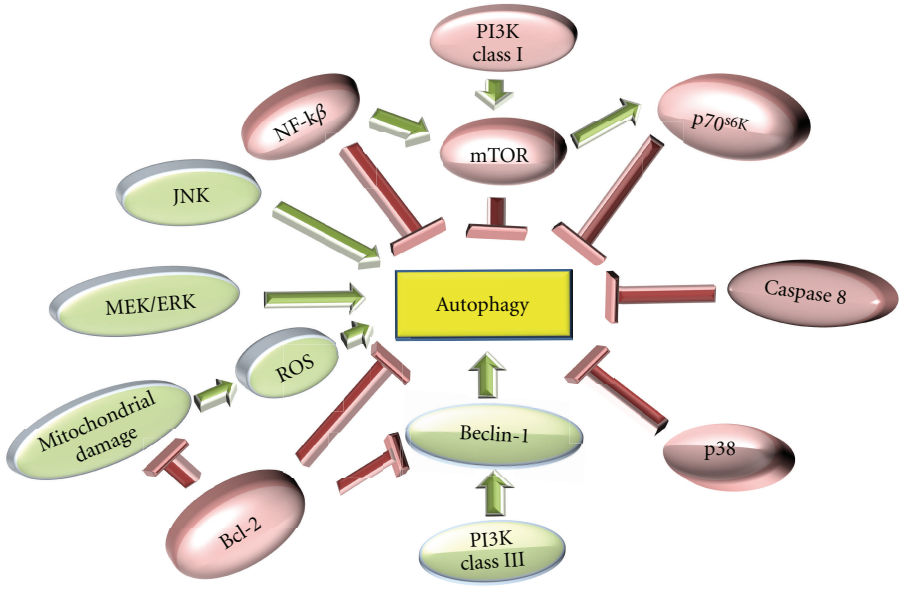

Figure 3: Molecular regulation of autophagy. In the figure, the factors that stimulate autophagy (green) are JNK, ERK1/2, ROS, or PI3K class III, whereas the inhibitory factors (red) are NF-κB, mTOR, caspase 8, Bcl-2, or p38.

the supply of nutrients is essential for cellular survival. In conditions of cellular nutrient deprivation, an increase in the levels of autophagy dependent on the inhibitory protein mTOR is induced to obtain energy by recycling the cell's own components. Many proteins participate in maintaining this equilibrium. The AMPK/mTOR/ULK1 route is one of the most widely studied pathways in terms of the cellular response to energy changes [67]. In the case of energy deficiency, the AMPK protein is responsible for inhibiting the TORC1 complex and activating the autophagy-initiating complex ULK1/Atg13/FIP200. Thus, AMPK participates directly in the regulation of autophagy by nutrient deprivation. It has been confirmed that LRRK2 and AMPK have a close relationship and a Ca^{2+}-dependent ability to induce the accumulation of autophagosomes [64]. In addition, LRRK2 siRNA induces an increase in autophagic activity and prevents the cellular death that is caused when autophagy is inhibited, which occurs in states of energy deficiency [61]. Moreover, ULK1/2 is a protein that participates in the regulation of the initial phases of autophagy and has been identified to play a role in the interaction with LRRK2, which could be responsible for the increase in autophagy when an increase in LRRK2 kinase activity is present [17]. Therefore, it appears that the LRRK2 protein can truly intervene in the regulation of the initial phases of autophagy and the induction of autophagy via nutrient deprivation.

4.2. Regulation of Nonclassic Autophagy Independent of Beclin-1. Alternative mechanisms of autophagy induction have been studied in which the classic autophagy protein Beclin-1 does not actively participate. The autophagy observed after treatments with MPTP corresponds to this pattern of autophagy independent of Beclin-1, as it has been observed that the autophagy does not revert after the use of Beclin-1 siRNA [68]. Furthermore, it has been demonstrated that MPTP provokes an increase in the expression of LRRK2 in neurons in the striatum [25], which could be related to an increase in autophagic activity of the cells after treatment with MPTP. However, there are contradictory results, as some studies have shown that the inhibition of this nonclassic autophagy independent of Beclin-1 protects the cell [39]. Others have indicated that the toxicity did not depend on or exacerbate the autophagy arising from increased LRRK2 expression, as there was no significant difference in the sensitivity to MPTP between wild type and LRRK2 knockout mice [69]. Therefore, further studies are needed to elucidate the relationship between the increase in LRRK2 protein expression and Beclin-1 independent autophagy and to identify how this relationship can influence the sensitivity of the cells.

4.3. Regulation of the Stability of the Cytoskeleton by LRRK2 and Its Importance in Autophagy. Studies focusing on the control of the quality of material that is degraded by autophagy have revealed the importance of proteins such as HDAC6 and actin for the maturation and completion of autophagy [70]. Many studies focused on the role of LRRK2 in the reorganization and functional stability of the cytoskeleton. LRRK2 phosphorylates proteins directly, such as heterotetramers of α/β-tubulin [30] and actin [31] or indirectly, such as moesin [71], ezrin, and radixin [72]. These proteins are essential for the regulation of actin activity, which suggests that LRRK2 is a regulator of cytoskeletal stability and an essential factor for efficient autophagy. One recent study indicated that the overexpression of Rac1 attenuated the disassembly of the actin filaments in cells

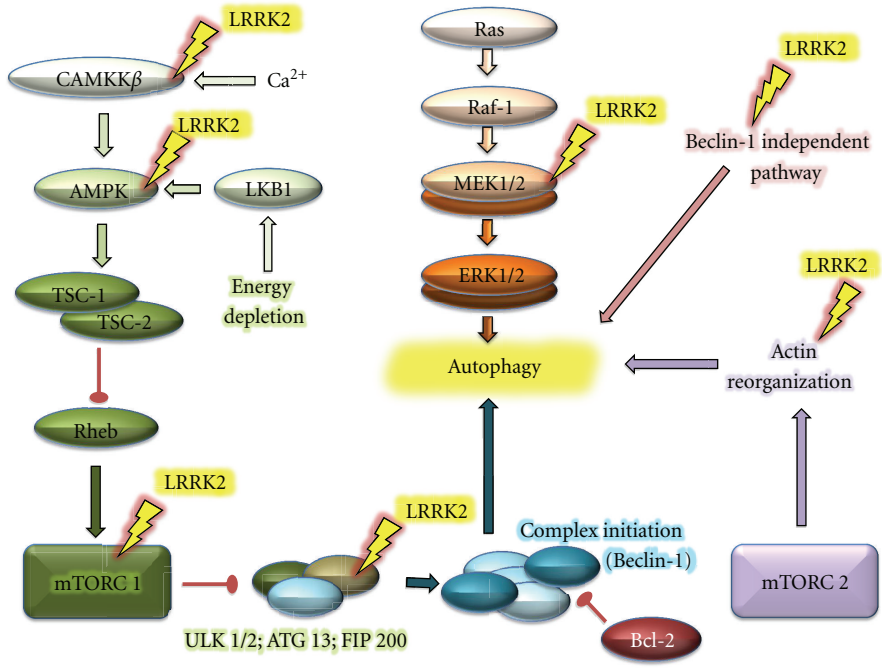

Figure 4: Diagram with the possible sites of interaction LRRK2-autophagy. The figure shows the different routes involved in the regulation of autophagy that may be LRRK2 dependent.

with G2019S mutations of LRRK2 [73], which supports the importance of LRRK2 activity in the correct assembly of the cytoskeleton.

4.4. Regulation of Autophagy Mediated by the MAPK p42/44 Pathway. MAPKs, JNK, and ERK1/2 are associated with positive regulatory processes of autophagy [40, 59, 74]. Recently, MAPKs have been documented as LRRK2 substrates [75]. In fact, an increase in the levels of ERK1/2 activity has been observed in cells that overexpress LRRK2 or its mutant forms G2019S and R1441C [35]. Studies that utilized pharmacological MEK/ERK1/2 route inhibitors such as U0126 revealed that the inhibition of this pathway hinders neurite retraction and exacerbates autophagy in cells with the G2019S LRRK2 mutation [40, 42]. Moreover, the sensitivity of cells is increased by the G2019S mutation when an increase in oxidative stress is present; this greater toxicity can be reverted through the use of the pharmacological MEK/ERK1/2 route inhibitor U0126 [34]. For this reason, the exacerbated autophagy that is produced by increased kinase activity of LRRK2, in which the MAPK ERK1/2 pathway actively participates, can be detrimental to the cell by increasing its sensitivity to oxidative stress [40]. In this sense many studies show that *G2019S* LRRK2 mutation induces alpha-synuclein aggregation, initiating and enhancing the formation of alpha-synuclein aggregates [76]. Moreover, this interaction is MEK/ERK pathway dependent [35], although this mechanism still remains unknown [74, 77–79]. Therefore, the defensive or protective autoregulatory mechanism that accelerates the degradation of misfolded proteins may explain the increased number of autophagic vacuoles in the brains of PD patients [80] and is possible than these exacerbated levels to be a critical contributing factor in the induction of cell death [81].

5. Conclusions and Future Perspectives

There is evidence of deregulated autophagy processes in neurons of the substantia nigra in PD patients. Thus, it is logical that deregulation could intervene, at least in part, in the etiology of PD [82]. The deregulation of autophagy has been associated with the LRRK2 protein. Deregulation is usually associated with the modulation of the activities of the protein, especially kinase activity. Some studies also indicate that the inhibition of LRRK2 kinase activity can protect against neuronal toxicity created by the *G2019S* mutation of LRRK2 [83], which is also responsible for the increase in autophagy levels. Furthermore, studies have indicated that LRRK2 is essential for the development of effective autophagy (Figure 4), as it is directly related to the cytoskeleton and cell membranes. Therefore, alterations in the kinase activity could deregulate this cell degradation mechanism and become toxic to the cell. Finally, LRRK2 could be involved in cell autophagy in response to stimuli such as deprivation, the generation of ROS, or drugs such as MPTP by making cells with LRRK2 dysfunction more sensitive to these stimuli.

LRRK2 protein is involved in cellular autophagy through direct modulation, the alteration of its own kinase activity, or the mediation of autophagy in response to external stimuli. The LRRK2 protein is also essential for maintaining the equilibrium between cellular degradation and synthesis.

Therefore, it is important to understand the activity of LRRK2 to elucidate the cellular death that has been identified in studies of PARK8 mutations. This knowledge is essential for the development of strategies for reducing the cellular sensitivity and cell death that could trigger the development of PD.

Authors' Contribution

These authors contributed equally to this paper.

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Review Article

Dopamine Oxidation and Autophagy

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The molecular mechanisms involved in the neurodegenerative process of Parkinson's disease remain unclear. Currently, there is a general agreement that mitochondrial dysfunction, α-synuclein aggregation, oxidative stress, neuroinflammation, and impaired protein degradation are involved in the neurodegeneration of dopaminergic neurons containing neuromelanin in Parkinson's disease. Aminochrome has been proposed to play an essential role in the degeneration of dopaminergic neurons containing neuromelanin by inducing mitochondrial dysfunction, oxidative stress, the formation of neurotoxic α-synuclein protofibrils, and impaired protein degradation. Here, we discuss the relationship between the oxidation of dopamine to aminochrome, the precursor of neuromelanin, autophagy dysfunction in dopaminergic neurons containing neuromelanin, and the role of dopamine oxidation to aminochrome in autophagy dysfunction in dopaminergic neurons. Aminochrome induces the following: (i) the formation of α-synuclein protofibrils that inactivate chaperone-mediated autophagy; (ii) the formation of adducts with α- and β-tubulin, which induce the aggregation of the microtubules required for the fusion of autophagy vacuoles and lysosomes.

1. Dopamine Synthesis and Degradation

Dopamine is a neurotransmitter that plays an essential role in the control of movements and loss of dopaminergic neurons containing neuromelanin in the nigrostriatal system. In addition, dopamine is involved in the development of motor symptoms experienced in patients diagnosed with Parkinson's disease (PD). Dopamine is synthesized in a sequential reaction in which the cytosolic enzymes tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC) catalyze the hydroxylation of the amino acid tyrosine to L-dihydroxyphenylanaline (L-dopa) and decarboxylation of L-dopa to dopamine, respectively. The protons of the hydroxyl group in dopamine dissociate when dopamine is localized in the cytosol at physiological pH. However, these protons are tightly bound to the hydroxyl group once dopamine is inside monoaminergic synaptic vesicles, which have a relatively low pH. The membrane of monoaminergic synaptic vesicles contains a vesicular monoaminergic transporter-2 (VMAT-2) that catalyzes the uptake of dopamine into these vesicles. These monoaminergic synaptic vesicles contain an ATPase that hydrolyzes ATP to ADP and Pi, and one proton (H^+) is translocated into the vesicle, generating a proton gradient. VMAT-2 uses this proton gradient to take up one molecule of dopamine with the concomitant release of two protons [1, 2]. The increase of protons inside monoaminergic synaptic vesicles induces a decrease in the pH of the vesicle, which is estimated to be 2 to 2.4 pH units lower than that of the cytosol [3]. TH and AADC have been shown to associate with monoaminergic synaptic vesicles containing VMAT-2 [4] by forming a complex. Tyrosine is then converted to L-dopa and immediately decarboxylated to dopamine, preventing the presence of free dopamine in the cytosol (Figure 1).

Dopamine in the cytosol spontaneously oxidizes to aminochrome without metal-ion catalysis [5]. Thus, VMAT-2 plays an important role in preventing the oxidation of dopamine in dopaminergic neurons. Other enzymes that prevent dopamine oxidation to aminochrome are monoamino oxidase (MAO) and catechol ortho-methyl transferase (COMT). MAO degrades excess dopamine in the cytosol by catalyzing the oxidative deamination of the amino group of dopamine to 3,4-dihydroxyphenylacetaldehyde with the concomitant formation of an ammonium molecule and hydrogen peroxide. Aldehyde dehydrogenase can then convert 3,4-dihydroxyphenylacetaldehyde to 3,4-dihydroxyphenylacetic acid (DOPAC), which can be

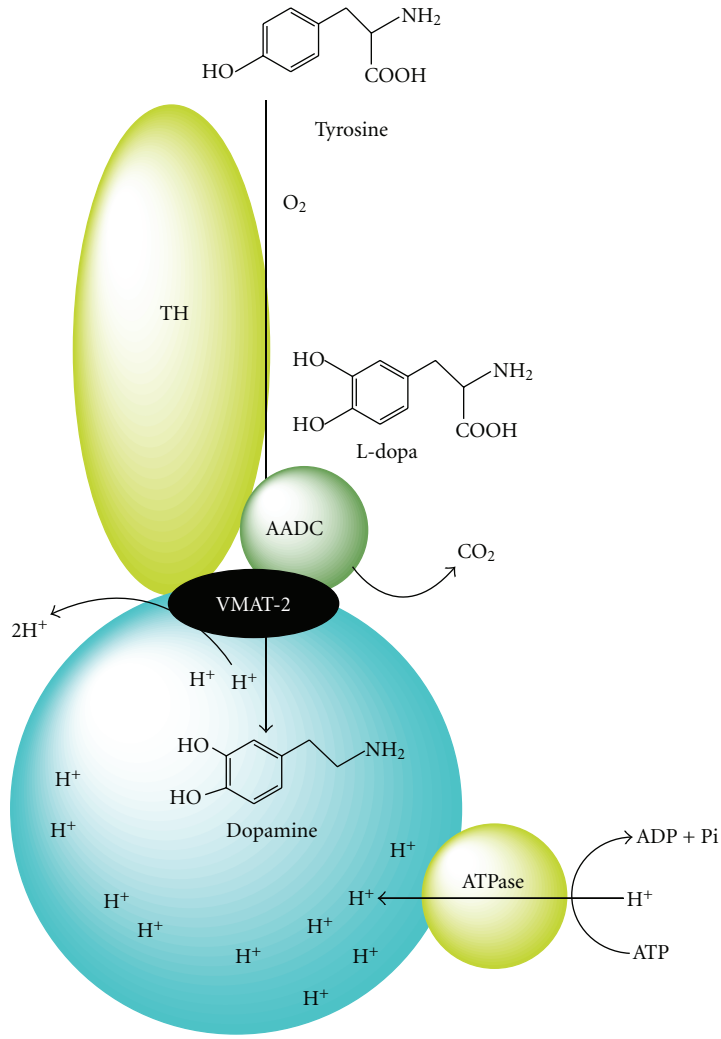

Figure 1: Dopamine synthesis. Synthesis of dopamine catalyzed by tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC), which are both associated with the vesicular monoaminergic transporter-2 (VMAT-2).

converted to homovanillic acid catalyzed by COMT. Dopamine can also be methylated by COMT, generating 3-methoxytyramine, which can be converted to 3-methoxy-4-hydroxyphenylacetaldehyde, hydrogen peroxide, and NH_3 by the enzyme MAO. Finally, the enzyme aldehyde dehydrogenase catalyzes the conversion of 3-methoxy-4-hydroxyphenylacetaldehyde to homovanillic acid (Figure 2). MAO enzymes are localized in the outer membranes of mitochondria in neurons, glial cells, and other cell types [6, 7]. MAO-A is mostly localized in catecholaminergic neurons, whereas MAO-B is found in serotonergic and histaminergic neurons as well as astrocytes [8]. COMT has two isoforms, one soluble (S-COMT) and one membrane-bound (MB-COMT) isoform. Both isoforms are found in microglial, astroglial, and some neuronal cells, such as pyramidal neurons, cerebellar Purkinje and granular cells, and striatal spiny neurons [9]. However, dopamine still oxidizes to aminochrome, even in the presence of VMAT-2, MAO-A, and S-COMT, which prevent the existence of free dopamine in the cytosol. Aminochrome, the precursor to neuromelanin, is a dark pigment found in dopaminergic neurons localized in the substantia nigra.

2. Dopamine Oxidation

Free cytosolic dopamine has protons that dissociate from their corresponding hydroxyl groups, promoting the oxidation of dopamine to dopamine *o*-quinone. This oxidation can proceed via a one-electron oxidation of dopamine to form a dopamine *o*-semiquinone radical (reaction 1), which is subsequently oxidized to dopamine *o*-quinone (reaction 2) by reducing two molecules of oxygen to superoxide radicals. The dopamine o-semiquinone radical does not strongly react with oxygen, leading to the formation of leukoaminochrome o-semiquinone radical during a one-electron reduction of aminochrome [10]. Subsequently, two dopamine *o*-semiquinone radicals can disproportionate, generating one molecule of dopamine *o*-quinone and one molecule of dopamine (reaction 3). A two-electron oxidation of dopamine to dopamine *o*-quinone is catalyzed by

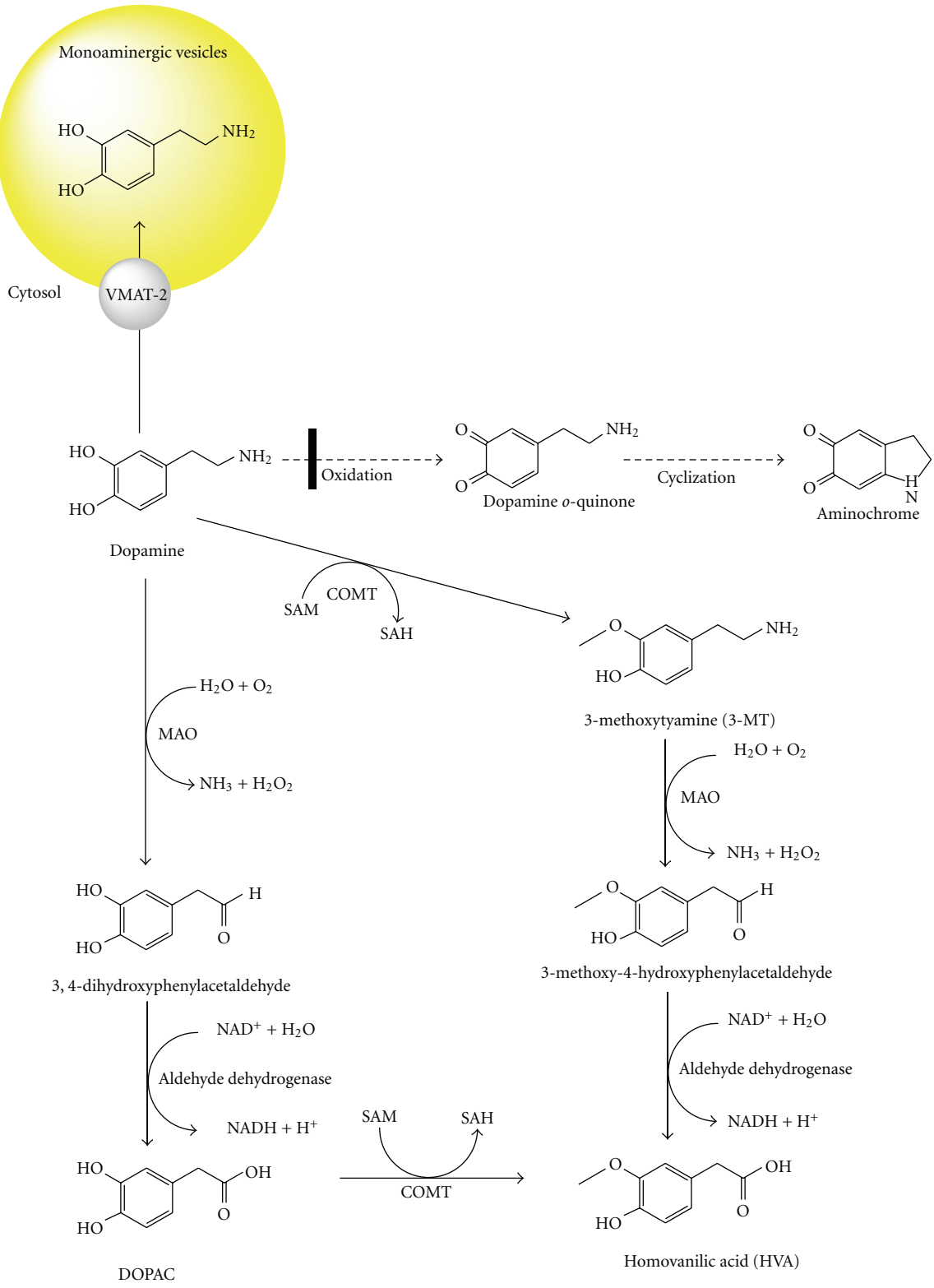

Figure 2: Dopamine degradation catalyzed by MAO and COMT. Dopamine oxidation to aminochrome is prevented by dopamine degradation mediated by both MAO and COMT. MAO catalyzes the oxidative deamination of dopamine amino group to 3,4-dihydroxyphenylacetaldehyde, that is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) catalyzed by aldehyde dehydrogenase. COMT catalyzes the methylation of dopamine to 3-methoxytyramine that is substrate for MAO that catalyzes the formation of 3-metoxy-4-hydroxyphenylacetaldehyde. Homovanillic acid is formed when MAO uses 3-metoxy-4-hydroxyphenylacetaldehyde as substrate or when DOPAC is metabolized by COMT.

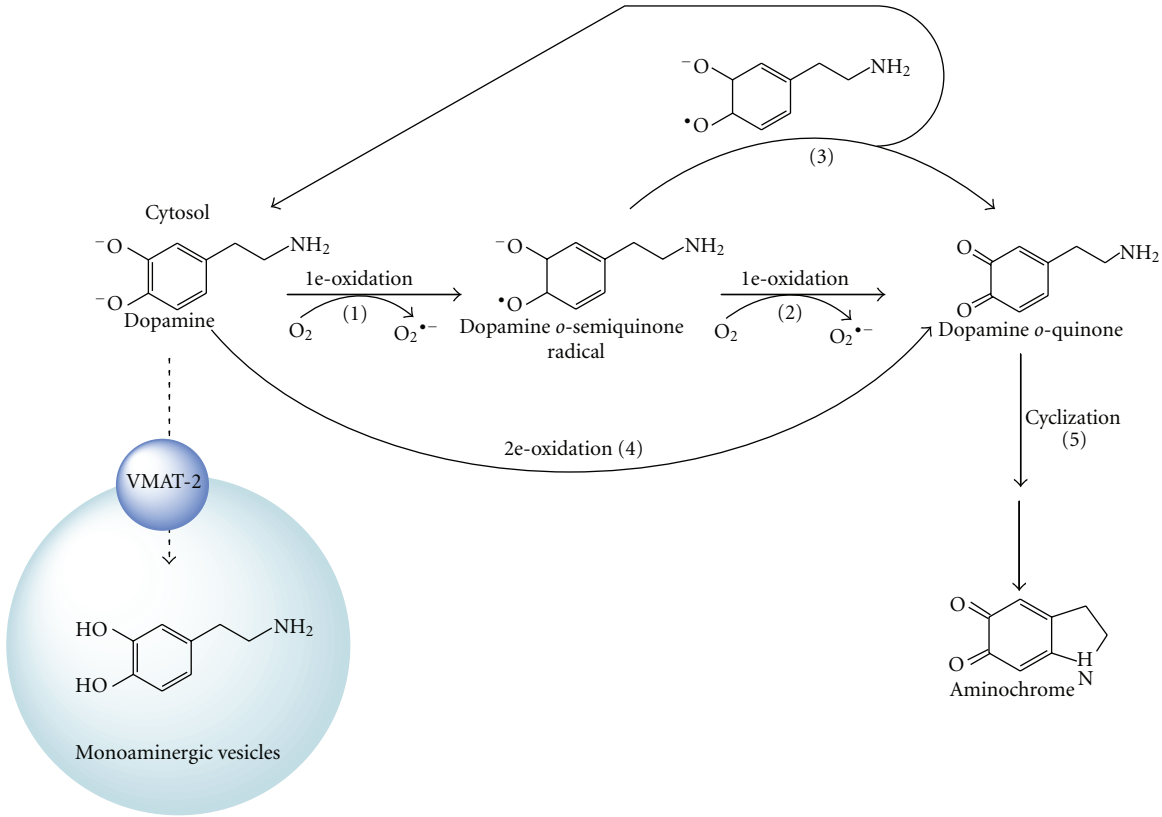

Figure 3: Dopamine oxidation to aminochrome at physiological pH. Dissociated dopamine can be oxidized to a dopamine *o*-semiquinone radical by the reduction of one molecule of oxygen to form a superoxide radical. The dopamine *o*-semiquinone radical can then disproportionate with another dopamine *o*-semiquinone radical, generating one molecule of dopamine and one molecule of dopamine *o*-quinone. Alternatively, the dopamine o-semiquinone radical can undergo one-electron oxidation to dopamine *o*-quinone by reducing one molecule of molecular oxygen to a superoxide radical. Dopamine *o*-quinone immediately cyclizes to form aminochrome, which is only stable in environments below pH 2.

the enzyme tyrosinase. Notably, the presence of dopamine *o*-semiquinone is not detected by electron spin resonance [11]. Dopamine *o*-quinone is not stable in the cytosol at physiological pH and its amino chain cyclizes (reaction 5), generating aminochrome (Figure 3). Dopamine *o*-quinone has been reported to form adducts with parkin, mitochondrial complex I and III, and dopamine transporters [12–14]. However, the molecule actually that forms these adducts is aminochrome because dopamine *o*-quinone is only stable below pH 2.0 [11]. Aminochrome is formed by the oxidation of dopamine by tyrosinase, which can be further purified by chromatography, and it is stable for approximately 3 hours [15].

The oxidation of dopamine can also be catalyzed by enzymes with peroxidase activity, such as prostaglandin H synthase, cytochrome P450 forms, dopamine β-monooxygenase, and xanthine oxidase [16–20]. Lactoperoxidase catalyzes the one-electron oxidation of dopamine to a dopamine *o*-semiquinone radical, which was confirmed by electron spin resonance [10]. However, dopamine can also be oxidized by metals, such as manganese, copper, iron, or sodium metaperiodate [11, 21–24]. At physiological pH, dopamine o-quinone is a transient product because it is unstable above pH 2, resulting in the further oxidation of dopamine [11]. Dopamine *o*-quinone rearranges by cyclizing its amino chain to form aminochrome (reactions 5 and 6). These proteins are inactivated by aminochrome because dopamine *o*-quinone cyclizes immediately at physiological pH [11].

3. Aminochrome Metabolism

3.1. Formation of Neuromelanin. Aminochrome is the precursor to neuromelanin because neuromelanin formation is dependent on the rearrangement of aminochrome to 5,6-dihydroxyindole, which is then oxidized to 5,6-indolequinone followed by further polymerization to form neuromelanin [25] (Figure 4). Postmortem studies using healthy subjects have shown that neuromelanin formation is a normal process in substantia nigra. Furthermore, this pigment is located in intact dopaminergic neurons because it is formed during the overtime and accumulates with age [26]. Neuromelanin acts as a chelator for metals [27, 28], indicating that this molecule plays a neuroprotective role. Neuromelanin accumulates in double membrane vacuoles, preventing neurotoxic effects of free neuromelanin in cells exposed to this pigment [29, 30].

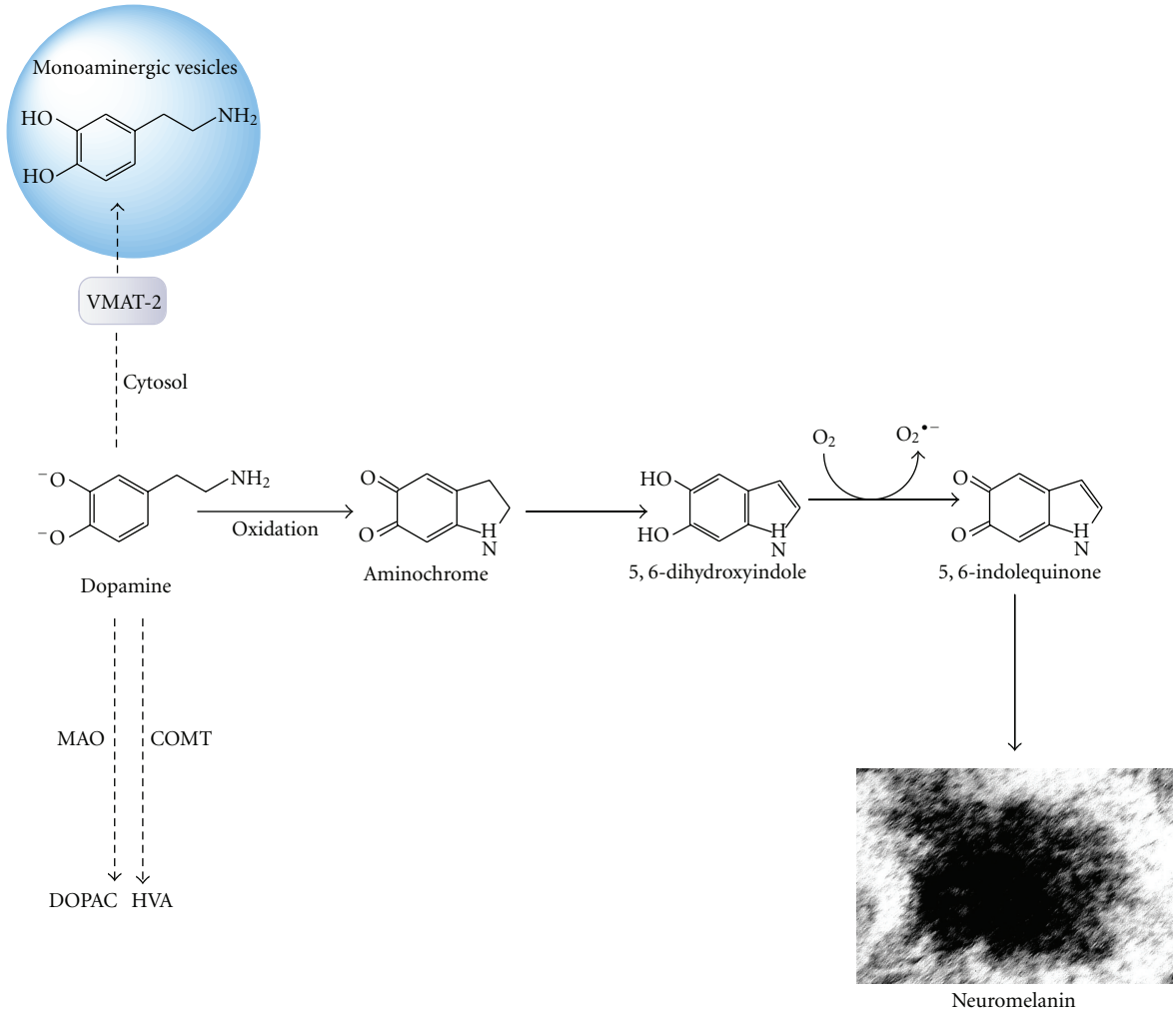

Figure 4: Neuromelanin formation. Dopamine is oxidized to aminochrome, which tautomerizes to 5,6-indolequinone and undergoes polymerization to form the dark pigment neuromelanin.

3.2. Formation of Aminochrome and Protein Adducts. Aminochrome forms adducts with proteins such as α-synuclein [31], stabilizing and inducing the formation of neurotoxic protofibrils [32]. In familiar PD, the formation of neurotoxic α-synuclein protofibers is dependent on a specific point mutation [33]. However, in sporadic PD, the formation of neurotoxic protofibrils appears to be dependent on the ability of aminochrome to form α-synuclein protofibrils. Aminochrome is also able to form adducts with mitochondrial complexes I and III, as well as isocitrate dehydrogenase [34], suggesting that this molecule induces mitochondrial dysfunction and a subsequent collapse in energy. Aminochrome also forms adducts with the protein DJ-1 [34], which has been suggested to be involved in the regulation of mitochondrial dynamics. Overexpression of the DJ-1 mutant associated with PD induces a significant increase in fragmented mitochondria, mitochondrial dysfunction, and increased neuronal vulnerability to oxidative stress [35].

Aminochrome has been reported to disrupt the architecture of the cytoskeleton in cell cultures [15] by forming aggregates with actin and α- and β-tubulin. Other studies also report the formation of aminochrome adducts with actin and β-tubulin [34]. In addition, aminochrome has been shown to form adducts with the ubiquitin carboxy-terminal hydrolase isoenzyme L1 (UCH-L1) [34], which was determined to be associated with familiar PD by a gene mutation (Figure 5).

3.3. One-Electron Reduction of Aminochrome. Aminochrome can undergo a one-electron reduction by flavoenzymes that utilize NADH or NADPH as an electron donator to generate a leukoaminochrome-*o*-semiquinone radical. This radical is extremely reactive with oxygen and autoxidizes to aminochrome under aerobic conditions. Molecular oxygen is then reduced to superoxide radicals, generating a redox cycle between the leukoaminochrome *o*-semiquinone radical and aminochrome [10, 36]. The redox cycling between aminochrome and leukoaminochrome *o*-semiquinone radical plays an important role in aminochrome neurotoxicity because it induces an energy collapse when flavoenzymes utilize NADH, which is required for ATP synthesis in

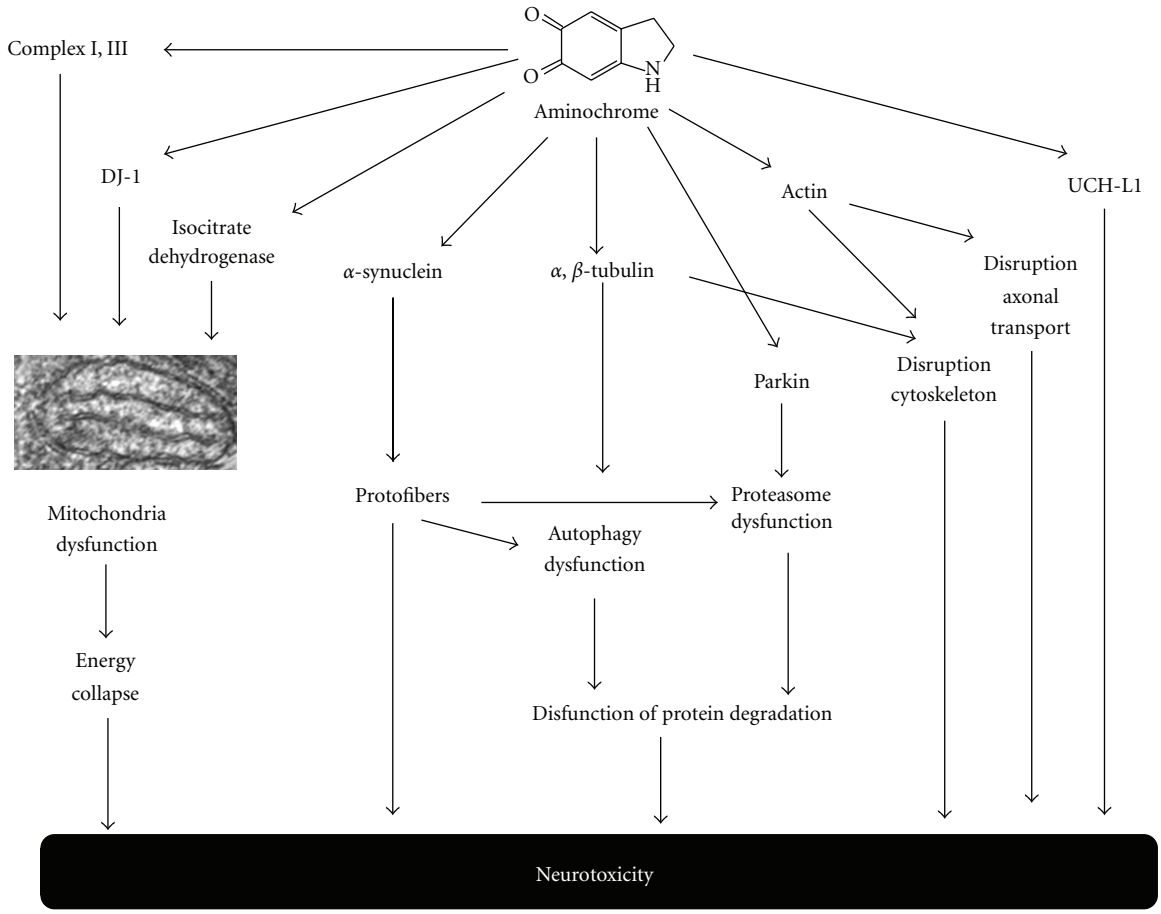

Figure 5: Aminochrome forms adducts with proteins. Aminochrome forms adducts with proteins, such as DJ-1, isocitrate dehydrogenase, and complex I and III of mitochondria, inducing mitochondrial dysfunction and an energy collapse. Adducts formed with α-synuclein, induces the formation of neurotoxic α-synuclein protofibrils, which inactivate chaperone-mediated autophagy and impair the proteasomal system, resulting in the dysfunction of protein degradation. Aminochrome adducts formed with α- and β-tubulin induces the aggregation of microtubules that are required for the fusion of autophagy vacuoles with lysosomes. Aminochrome also forms adducts with UCHL-1.

the mitochondria [37, 38]. The use of NADPH in redox cycling also affects the cell because NADPH is required to catalyze the reduction of oxidized glutathione by glutathione reductase, which is an important antioxidant. The neurotoxic effects of this redox cycling are enhanced by the dismutation of superoxide radicals to hydrogen peroxide, the precursor of hydroxyl radicals. The one-electron reduction of aminochrome has been reported to be neurotoxic to catecholaminergic cells [15, 21, 22, 38–45] (Figure 6).

3.4. Two-Electron Reduction of Aminochrome. Aminochrome can undergo a two-electron reduction by DT-diaphorase (EC.1.6.99.2), a flavoenzyme that uses both NADH and NADPH as electron donors, and the product of this reaction is the hydroquinone leukoaminochrome [11]. Leukoaminochrome can autoxidize in the presence of superoxide radicals. However, the presence of superoxide dismutase in the cytosol prevents leukoaminochrome autoxidation from occurring [36]. We have proposed a protective role for DT-diaphorase against aminochrome neurotoxicity, which is supported by cell culture studies based on the inhibition of DT-diaphorase with dicoumarol and the induced aminochrome neurotoxicity that results from reduced DT-diaphorase expression by siRNA [38, 41, 42, 46]. DT-diaphorase also prevents the formation of α-synuclein protofibrils [47, 48] and disruption of the cytoskeleton, which is generally the consequence of forming adducts with actin and α- and β-tubulin [15]. DT diaphorase immunoreactivity has been observed in dopaminergic neurons and Bergmann glia, astrocytes, and tanycytes [49] (Figure 7).

3.5. Aminochrome Conjugation with Glutathione. Aminochrome can be conjugated with glutathione by glutathione S-transferase M2-2 (GST M2-2) to 4-*S*-glutathionyl-5,6-dihydroxyindoline. 4-*S*-Glutathionyl-5,6-dihydroxyindoline is stable in the presence of biological oxidizing agents, such as oxygen, superoxide radicals, and hydrogen peroxide [50, 51]. The stability of 4-*S*-glutathionyl-5,6-dihydroxyindoline in the presence of biological oxidizing agents suggest that is a final elimination product. Interestingly, the precursor of aminochrome, dopamine *o*-quinone, is also conjugated by GST M2-2 to 5-glutathionyl-dopamine, preventing the formation of aminochrome [52]. All glutathione conjugates undergo degradation of the tripeptide γ-L-Glu-L-Cys-Gly,

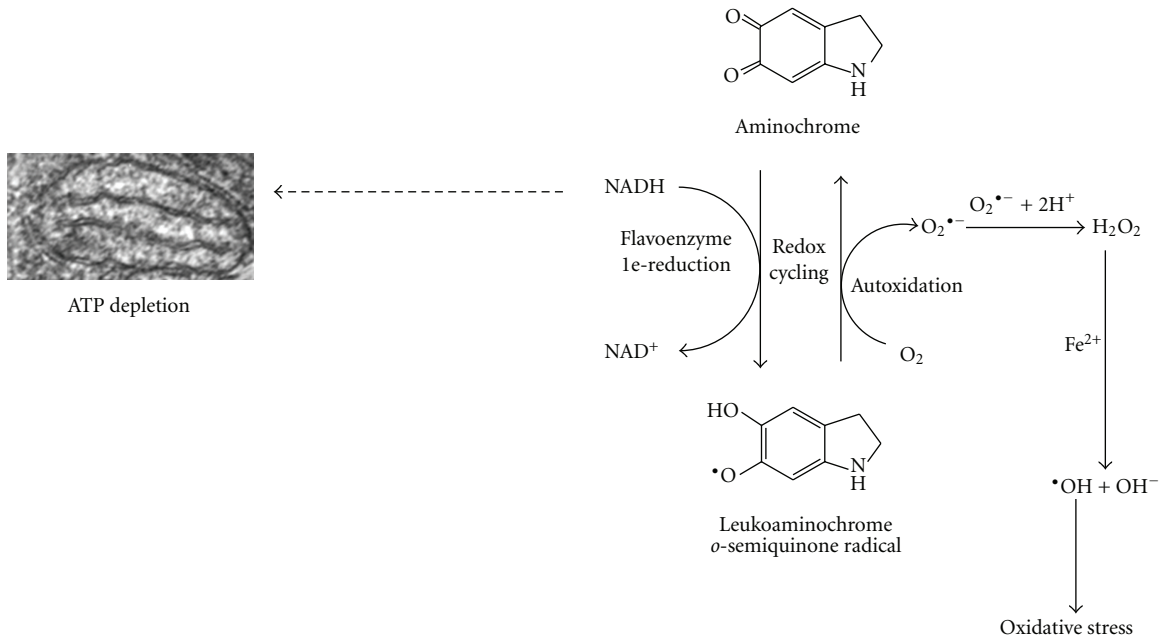

Figure 6: One-electron reduction of aminochrome. The one-electron reduction of aminochrome is catalyzed by flavoenzymes that utilize NADH as an electron donor, generating leukoaminochrome *o*-semiquinone radical. This radical is extremely reactive with oxygen and autoxidizes by reducing molecular oxygen to superoxide radicals. This redox cycling induces oxidative stress and depletes the NADH required for the generation of energy (ATP) in the mitochondria. Redox cycling can also be catalyzed by flavoenzymes that utilize NADPH as an electron donor, depleting the NADPH required for the reduction of oxidized glutathione.

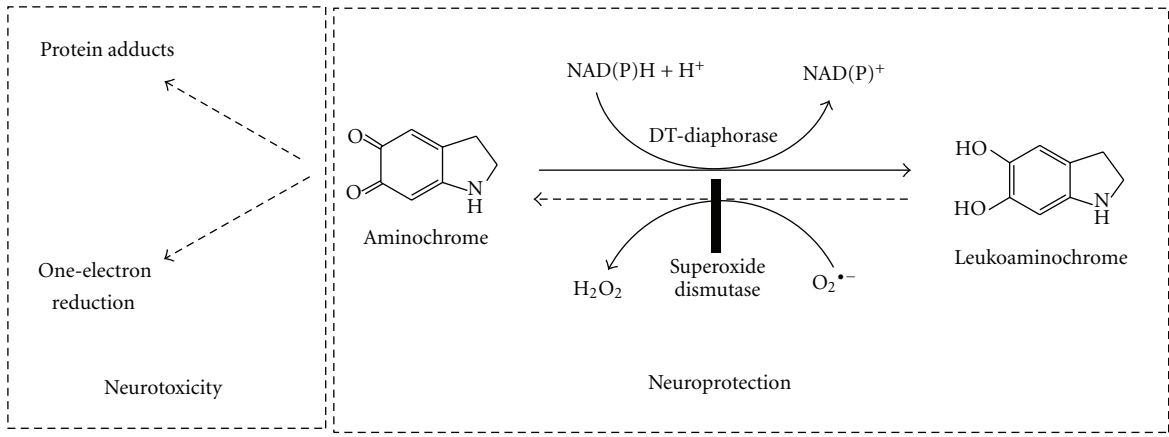

Figure 7: Two-electron reduction of aminochrome catalyzed by DT-diaphorase. DT-diaphorase prevents the participation of aminochrome in neurotoxic reactions, such as the formation of adducts with proteins and one-electron reduction of aminochrome to leukoaminochrome.

named the glutathione to cysteil conjugate. Thus, 5-glutathionyl-dopamine is converted to 5-cysteinyl dopamine. Notably, 5-S-cysteinyl-dopamine has been detected in the cerebrospinal fluid of PD patients, dopamine-rich regions of the brain such as the caudate nucleus, putamen, globus pallidus and substantia nigra, and in neuromelanin [53–55]. GST M2-2 also catalyzes the conjugation of dopa *o*-quinone to 5-glutathionyl dopa leads to the degradation of the tripeptide glutathione, generating 5-cysteinyl dopa [56]. Melanoma cells produce and release 5-cysteinyl-dopamine, which is then excreted through the urine [57]. Thus, the conjugation of glutathione must be a protective reaction against aminochrome neurotoxicity (Figure 8).

4. PD and Autophagy

Autophagy is an important intracellular bulk degradation and recycling process in which cytoplasmic proteins and organelles accumulate in autophagy vacuoles that are transported into lysosomes [58–60]. Autophagy plays an important role in the elimination of damaged organelles, such as the mitochondria. Autophagy dysfunction has been speculated to play an important role in the pathogenesis of PD [61]. Wild-type α-synuclein is degraded by chaperone-mediated autophagy (CMA) and macroautophagy because the inhibition of CMA and macroautophagy lead to accumulation of wild type α-synuclein [62]. The expression of

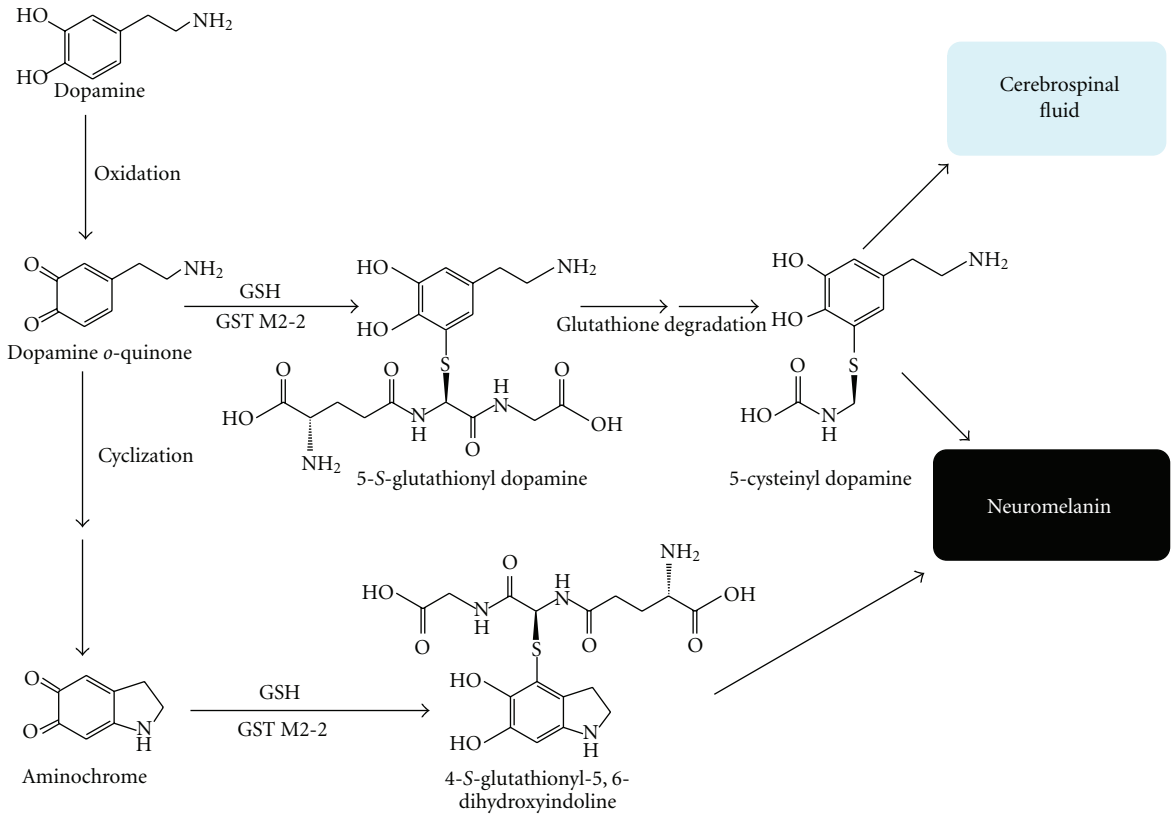

Figure 8: Glutathione conjugation of dopamine *o*-quinone and aminochrome. GST M2-2 catalyzes conjugation of both aminochrome and its precursor dopamine *o*-quinone to 4-*S*-glutathionyl-5,6-dihydroxyindoline and 5-glutathionyl-dopamine, respectively. 5-Glutathionyl-dopamine undergoes degradation to 5-cysteinyl dopamine that have been found in both the cerebrospinal fluid and neuromelanin.

the α-synuclein mutant A53T induces CMA dysfunction, which is mediated by the expression of α-synuclein protofibrils [63]. Interestingly, the pathogenic A53T and A30P α-synuclein mutants inhibit their own degradation and that of other substrates [64]. Overexpression of the α-synuclein mutant A53T in transgenic animals demonstrates that A53T localizes to mitochondrial membranes as a monomer, inhibiting complex I and increasing mitochondrial autophagy [65]. α-synuclein impairs autophagy via Rab1a inhibition, and Rab1a overexpression rescues the autophagy defect caused by α-synuclein [66]. A mutation in ubiquitin C-terminal hydrolase L1 (UCH-L1), which is associated with familial PD, was reported to inhibit CMA autophagy by interacting with the lysosomal receptor of CMA LAMP-2A [67]. Parkin has also been shown to promote autophagy of damaged mitochondria by relocalizing into dysfunctional mitochondria with low membrane potentials in mammalian cells [68]. The loss of DJ-1 induces a reduction in the mitochondrial membrane potential and an increase in the fragmentation and accumulation of autophagy markers. These effects appear to be mediated by oxidative stress because supplementing DJ-1-deficient cells with glutathione has been shown to reverse these effects on mitochondria and autophagy [69]. Transfection of the common G2019S LRRK2 mutation into SH-SY5Y cells was reported to increase autophagy in both neuritic and somatic compartments [70]. Autophagy activation was observed to restore the mitochondrial membrane potential impaired by rotenone in SH-SY5Y cell lines overexpressing α-synuclein [71] and attenuate rotenone-induced toxicity in SH-SY5Y cell lines [72].

5. Aminochrome and Autophagy

In Parkinson's disease, autophagy dysfunction plays an important role in the neurodegeneration of dopaminergic neurons containing neuromelanin. Proteins associated with familial PD have been reported to play a role in autophagy dysfunction, such as α-synuclein, UCH-L1, and DJ-1 [63, 67, 69]. Mutated α-synuclein (A53T) generates protofibrils that inhibit CMA autophagy. As a result, dopamine-modified α-synuclein is poorly degraded by CMA and also inhibits the degradation of other substrates using this pathway [73]. Aminochrome was reported to form adducts with α-synuclein by binding to the 125YEMPS129 motif of α-synuclein and inducing and stabilizing the formation of protofibrils [31]. These observations suggest that aminochrome is involved in the alpha synuclein-dependent inhibition of CMA autophagy because aminochrome induces the formation of α-synuclein protofibrils, such as the A53T mutant (Figure 10). Mutated UCH-L1 also inhibits CMA autophagy by interacting with the lysosomal receptor for CMA LAMP-2A [67]. Aminochrome has been shown to also form adducts with UCH-L1 [34]. Little is known about the

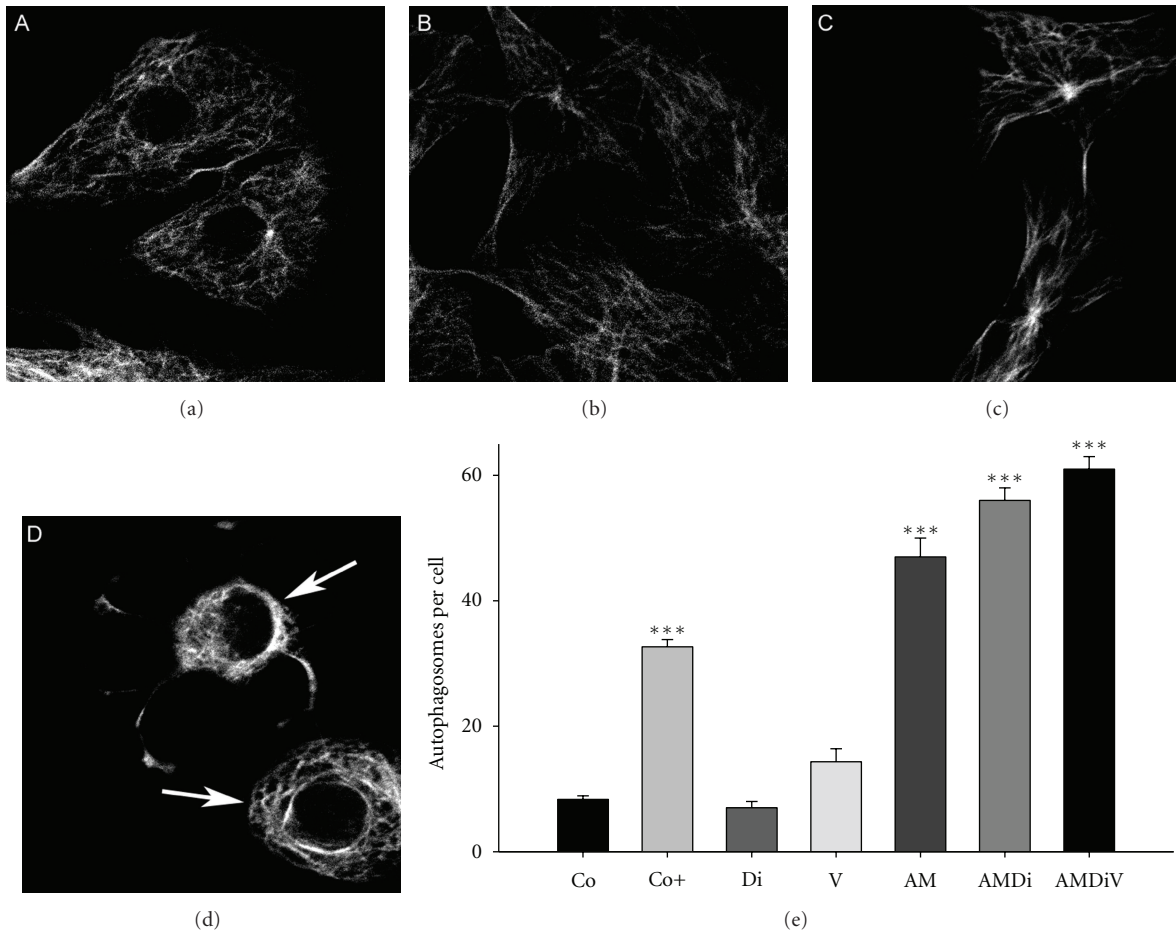

Figure 9: The effects of aminochrome on β-tubulin (a–d) and the accumulation of autophagosomes in the cytosol of cells treated with aminochrome (e). RCSN-3 cells were treated with cell culture medium (a), 100 μM dicoumarol (b), 50 μM aminochrome (c), and 50 μM aminochrome and 100 μM dicoumarol (d) and incubated for 48 h as described by Paris et al. 2010 [15]. In (e) the number of autophagosomes per cell was determined by incubating RCSN3 cells for 24 h with cell culture medium (Co), 100 μM dicoumarol (Di), 10 μM vinblastine (V), 20 μM aminochrome (AM), 20 μM aminochrome and 100 μM dicoumarol (AMDi), 20 μM aminochrome and 100 μM dicoumarol and 10 μM vinblastine (AMDiV). As a positive control, cells were incubated with cell culture medium without bovine serum (Co+). The statistical significance was assessed using ANOVA for multiple comparisons and Student's t test ($^{***}P < 0.001$). This experiment was performed as described by Paris et al. 2011 [40].

effects of the aminochrome-induced modification of UCH-L1. However, we speculate that the aminochrome-induced modification of UCH-L1 also impairs CMA autophagy. In addition, aminochrome forms adducts with the protein DJ-1, and the loss of DJ-1 indirectly alters autophagy by interfering with the regulation of oxidative stress [69].

Microtubules are an important component of the cytoskeleton, which are composed by subunits of α- and β-tubulin and normally exist as dimers. Microtubules also play a role in the formation of autophagosomes and fusion of autophagosomes with lysosomes [74, 75]. Aminochrome forms adducts with β-tubulin [34] and the aminochrome one-electron reduction product when DT-diaphorase is inhibited by dicoumarol. This inhibition leads to the disruption of the cytoskeleton by disrupting the α- and β-tubulin network and its aggregation around the cell membrane ([15]; Figure 9). Thus, we speculate that aminochrome prevents the fusion of autophagosomes with lysosomes by inducing the aggregation of microtubules. We hypothesize that the number of autophagosomes will increase in the cytosol when aminochrome inhibits the fusion between autophagosomes and lysosomes by preventing the formation of normal microtubules because of the formation of aminochrome adducts with α- or β-tubulin (Figure 10). Incubation of RCSN-3 cells with 20 μM aminochrome in either the presence or absence of 100 μM dicoumarol induced a significant increase in the number of autophagosomes in the cytosol (6- and 9-fold, resp.; Figure 9). These results support aminochrome playing a role in autophagy dysfunction. Aminochrome forms adducts with α- or β-tubulin, preventing the fusion of autophagosomes and lysosomes that lead to an increase in the number of autophagosomes in the cytosol [15]. Furthermore, these data support the observation that aminochrome induces a significant increase of GFP-LC3 positive staining in cells treated with aminochrome [41]. Aminochrome has also been reported to inactivate parkin, an ubiquitin ligase

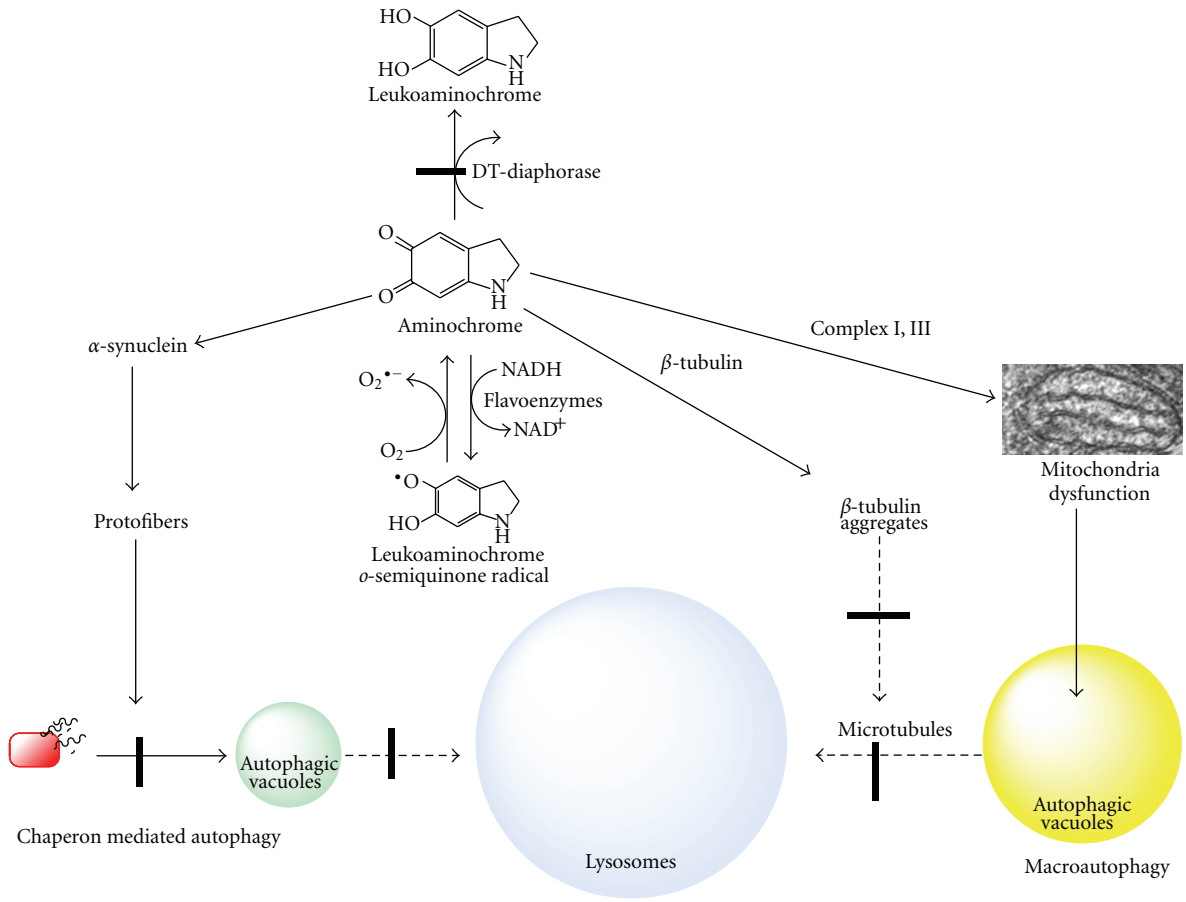

Figure 10: Possible role of aminochrome in autophagy. Aminochrome induces the dysfunction of autophagy by forming adducts with the following: (i) alpha synuclein, which inactivates chaperone-mediated autophagy; and (ii) α- and β-tubulin, inducing the aggregation of microtubules, which are essential for the fusion of autophagy vacuoles with lysosomes.

of the proteasomal system, by forming adducts with parkin [14] as well as inhibit the proteasome [76]. All of these results support the involvement of aminochrome in the dysfunction of protein degradation.

Acknowledgments

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Review Article

Mitochondrial Dynamics and Mitophagy in the 6-Hydroxydopamine Preclinical Model of Parkinson's Disease

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We discuss the participation of mitochondrial dynamics and autophagy in the 6-hydroxidopamine-induced Parkinson's disease model. The regulation of dynamic mitochondrial processes such as fusion, fission, and mitophagy has been shown to be an important mechanism controlling cellular fate. An imbalance in mitochondrial dynamics may contribute to both familial and sporadic neurodegenerative diseases including Parkinson's disease. With special attention we address the role of second messengers as the role of reactive oxygen species and the mitochondria as the headquarters of cell death. The role of molecular signaling pathways, for instance, the participation of Dynamin-related protein 1(Drp1), will also be addressed. Furthermore evidence demonstrates the therapeutic potential of small-molecule inhibitors of mitochondrial division in Parkinson's disease. For instance, pharmacological inhibition of Drp1, through treatment with the mitochondrial division inhibitor-1, results in the abrogation of mitochondrial fission and in a decrease of the number of autophagic cells. Deciphering the signaling cascades that underlie mitophagy triggered by 6-OHDA, as well as the mechanisms that determine the selectivity of this response, will help to better understand this process and may have impact on human treatment strategies of Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is progressive neurodegenerative condition that is characterized by the presence of motor and nonmotor symptoms, of which the etiology remains poorly understood. Nevertheless, a broad range of studies conducted over the past few decades have collectively identified a number of molecular/cellular events that might underlie PD pathogenesis. In particular, the participation of mitochondrial-mediated pathways has provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration. Mitochondria can be considered as headquarters where the cell controls signaling pathways that under some circumstances can lead to cell death [1, 2]. Mitochondrial membrane permeabilization is a critical event during apoptosis and represents the point of no return of this lethal process [3]. For instance, the permeabilization of the mitochondrial outer membrane (MOMP), which allows the release of mitochondrial death factors, facilitates or triggers different signaling cascades that ultimately cause the execution of cell death. In many PD experimental models, including the addition of parkinsonian neurotoxins to cell cultures, the participation of MOMP has been described, resulting in the release of cytochrome c from mitochondria [4].

In the past, mitochondria have been suggested to be filamentous, rigid, and static organelles incrusted into the cytosol with the only function of being the main source or energy to the cell in the form of ATP. In fact, mitochondria are dynamic and mobile organelles that constantly undergo membrane remodeling through repeated cycles of fusion and fission. In addition, regulated turnover occurs via a

specialized lysosome-mediated degradation pathway known as "mitophagy," a term originally coined by Lemasters [5].

6-Hydroxydopamine (6-OHDA), also known as oxidopamine or 2,4,5-trihydroxyphenethylamine ($C_8H_{11}NO_3$), is a toxic oxidative metabolite of dopamine and is detected in the brains and urine of Parkinson's disease (PD) patients. It has been applied broadly to generate experimental models of Parkinson's disease. There is accumulating evidence from *in vitro* and *in vivo* studies, implicating cell death in the etiology of the 6-OHDA model of PD [6–9].

2. 6-OHDA and Mitochondrial Dynamics

The regulation of mitochondrial dynamics processes such as fusion, fission, and mitophagy, signifies an important mechanism controlling cellular fate [10]. Mitochondrial fission and fusion are antagonistic activities. Their fundamental roles are to create a compartment that is a connected conductor, which is able to mix its contents. Also, they function to have access to mtDNA and its products in order to be distributed to distant cellular destinations through transport via actin or microtubule networks. The importance of mitochondrial dynamics to cellular function is perhaps best appreciated in neurons. These postmitotic cells, particularly those with vast axonal field, require high energy to support their operations, which include the active transportation of components (including mitochondria) toward metabolically demanding synaptic terminals that are distally located.

An imbalance in mitochondrial dynamics may contribute to both familial and sporadic neurodegenerative diseases including PD [11–14]. Evidence exists suggesting that an amplification of fission events can cause pathogenesis of human PD. Stress stimuli that are used to study PD, such as rotenone [15], annonacin [16], and 6-OHDA [17], are capable of inducing mitochondrial fission. Also, human fibroblasts from PD patients exhibit elevated levels of fragmented mitochondria [18].

Evidence has been presented showing that tipping the equilibrium toward continuous mitochondrial fission can evoke a neurodegenerative cascade [19]. Intriguingly, inherited loss-of-function mutations of MFN2 or OPA1 cause progressive neuropathies in humans. MFN2 mutations cause Charcot-Marie-Tooth type 2A (CMT-2A), a peripheral neuropathy characterized by motor and sensory neuron loss [20]. OPA1 mutations cause autosomal dominant optic atrophy, which is characterized by retinal ganglion cell and optic nerve degeneration [21].

In the dopaminergic cell line SH-SY5Y, using immunofluorescence studies with antibodies raised against the mitochondrial matrix protein MnSOD, we have shown that in untreated cells mitochondria exhibited a predominantly elongated and filamentous morphology. Strikingly, after addition of 50 μM 6-OHDA mitochondria formed short and spherical structures, due to the fragmentation of single filamentous mitochondria into multiple isolated organelles [17]. Furthermore, time-lapse fluorescence microscopy revealed that 6-OHDA-induced mitochondrial fragmentation occurred rapidly and synchronous within 15 min after 6-OHDA addition and was visible in approximately 80% of the cells after 3 h. Thus, mitochondrial fragmentation appears to be an early event in 6-OHDA-induced cell death. Nevertheless, significant changes in the chromatin structure were not detected early on. 6-OHDA (50 μM) had to be present more than 9 h to initiate significant changes in mitochondrial membrane potential in SH-SY5Y cells, placing mitochondrial alterations in an early stage of 6-OHDA-activated pathways.

Mitochondrial fission is highly regulated process and is mediated by a defined set of proteins [22–25]. One of these proteins, Dynamin-related protein 1 (Drp1), is a member of the dynamin family of large GTPases and mediates the scission of mitochondrial membranes through GTP hydrolysis. Drp1 predominantly is a cytoplasmic protein and associates with mitochondrial fission sites upon oligomerization [26, 27]. How Drp1 mediates outer membrane scission is unclear but it has been proposed that, similar to Dynamin, it may act as a mechanoenzyme [28]. Upon activation by an unknown mechanism, Drp1 assembles via Fis1 [29] into large complexes at future scission sites (cut sites) on the inner mitochondrial membrane [30]. Overexpression of Fis1 can induce directly both mitochondrial fragmentation and apoptosis [29]. However, Fis1 or mitochondrial fission is not requisites for apoptosis since cytochrome c release is prevented in cells overexpressing Fis1 when proapoptotic Bax/Bak are inactivated [31, 32].

Indicating that mitochondrial fission process may be important for apoptosis, dominant-negative forms of Drp1 that antagonize mitochondrial division delay the release of cytochrome c and the onset of cell death [33], although, not as potently as some antiapoptotic Bcl-2 family members, such as Bcl-xL. Moreover, ectopic Mfn2, Opa1, and mutant forms of Opa1 can also confer protection against programmed cell death [34–36].

We have revealed that 6-OHDA requires the dynamin-like GTPase Drp1 to induce mitochondrial division. We have also observed that Drp1 translocated to mitochondria 3 h after addition of 50 μM 6-OHDA, although the levels of total Drp1 were unchanged in cellular extracts [37]. When SH-SY5Y cells were transfected with Drp1 siRNA duplexes to silence Drp1, 6-OHDA-induced mitochondrial fragmentation was inhibited. Furthermore, 6-OHDA-induced cell death was reduced after silencing of Drp1. In line with these findings, inhibition of Drp1 function in other experimental models has also been shown to prevent mitochondrial fission and cell death [33, 38, 39]. In a recent report, a block in mitochondrial fission by the expression of dominant-negative Drp1 or wild-type Mfn1 prevented mitochondrial fragmentation and rescued neurons from nitric- oxide- (NO-) induced degeneration and cell death.

Nowadays we have the pharmacological possibility of inhibiting Drp1 activity. For instance, Cassidy-Stone and colleagues [40] have identified an inhibitor of mitochondrial division, called mitochondrial division inhibitor-1 (mdivi-1), using yeast screens of chemical libraries. Mdivi-1 reduces mitochondrial fission after several insults [40, 41]. Mdivi-1 inhibits Dnm1 assembly and GTPase activity *in vitro*.

Examining the activity of a series of mdivi-1 analogs shows a correlation between the degree of inhibition of GTPase activity and the extent of inhibition of yeast mitochondrial fission. Recently, another group identified an inhibitor of Dynamin-1, Dynamin-2, and DRP1, called Dynasore, which binds the GTPase domain and inhibits GTPase activity [42]. Mdivi-1 appears to be more selective than Dynasore, as it affects neither the activity of the Dynamin-1 GTPase *in vitro* nor that of the two mitochondrial dynamin family members mediating yeast mitochondrial fusion, Fzo1 or Mgm1. This is because mdivi-1 does not inhibit mitochondrial fusion *in vivo*. This specificity has been proposed to stem from mdivi-1 binding outside the GTPase domain to a surface that is involved in oligomeric assembly, thereby inhibiting Dnm1/DRP1 GTPase activation. Mechanistically, mdivi-1 acts as a mixed-type inhibitor to attenuate the early stages of division DRP assembly by preventing the polymerization of higher-order structures. Mdivi-1 selectively targets the unassembled pool of the mitochondrial division dynamin, and its binding creates and/or stabilizes an assembly-deficient conformation [43]. Furthermore, inhibiting mitochondrial division with mdivi-1 in Parkinson's disease cell culture models or a dominant negative form of Drp1 in Alzheimer's and Huntington's disease cell culture models attenuates disease-associated phenotypes [44]. For a review see [43].

Alternatively, mitochondrial dynamics may be initiated by insertion of the protooncogene Bcl-2 family into the MOM. The Bcl-2 family is composed of about 25 key regulators of apoptotic processes. These proteins are structural and functional homologs of the nematode protein CED-9 and are localized in the mitochondrial membrane. They contain up to four regions with a high homology to Bcl-2 (regions BH 1 to 4) [45]. Members containing only the BH3 region are proapoptotic proteins, and among them are Bax (X Bcl-2-associated protein), Bak (Bcl-2-antagonist/killer), BIM, and BID. Inactive Bax resides in the cytosol or is anchored to the laxly face of the membranes of various organelles [46]. Recently, several members of Bcl-2 family, including both, pro- and antiapoptotic proteins, have been shown to play a role in mitochondrial morphogenesis in healthy cells [37, 47]. Finding that Bax and Bak promote mitochondrial fusion in healthy cells [47] was unanticipated, as Bax and Bak form foci that colocalize with ectopic Mfn2 and Drp1 at the sites of mitochondrial division to promote mitochondrial fission during apoptosis [47]. After a cell death signal, the Bax protein acquires a homooligomeric shape and is incorporated into the outer mitochondrial membrane. Postmortem studies indicated that the presence of Bax and its translocation to the outer mitochondrial membrane may contribute to the death of dopaminergic neurons in PD [48]. In addition, the proapoptotic Bax protein colocalized to scission sites on mitochondria, suggesting that the mitochondrial fission machinery cooperates with the cell death machinery [49]. We have shown that Bax actively participates in the 6-OHDA preclinical model of PD [4, 17]. Furthermore, in our experimental model, mitochondrial Bax translocation took place after mitochondrial fragmentation and Drp1 translocation. SH-SY5Y cells consistently showed mitochondrial Bax localization 6 h after 6-OHDA addition. We were unable to find mitochondrial Bax-aggregation loci at the very early time points where mitochondrial fragmentation was already evident (<3 h of treatment). On the other hand, 6-OHDA-induced mitochondrial Bax translocation was independent of Drp1 and mitochondrial fission. Thus, Mdivi-1 failed to abrogate the translocation of Bax to the mitochondria upon 6-OHDA additions. In agreement with this, in $Drp1^{-/-}$ cells [50] or in cells that were transfected with a dominant negative allele, DrpK38A, that is defective in GTP binding [33, 39, 49, 51], Bax translocates to the mitochondria with kinetics similar to those observed in wild-type cells.

In addition, several studies have reported that preventing mitochondrial fission during apoptosis leads to a partial inhibition of cytochrome c release [33, 35, 36, 38, 51]. Mitochondrial fission is not required for cell death. However, this does not exclude that fragmentation of the mitochondrial network might potentiate cell death.

Mitochondrial dynamics has also been proposed to play a role in the quality control of the organelle. During a division event, functionally asymmetric daughter mitochondria with different membrane potentials can be produced. The functional daughter, which retains a high membrane potential, can refuse with the mitochondrial network, whereas the dysfunctional daughter cannot refuse due to the low membrane potential and is subsequently flagged for autophagic degradation [52, 53].

3. 6-OHDA Inductors of Autophagy

The mitochondrial quality control hypothesis postulates that dysfunctional mitochondria are susceptible to degradation [53]. Autophagy is a stress-induced catabolic process involving the lysosome (or, in yeast, the analogous vacuole), which is conserved in all eukaryotes [54, 55]. According to the different pathways by which cargo is delivered to the lysosome or vacuole, autophagy is divided into three main types: chaperone-mediated autophagy, microautophagy, and macroautophagy [56]. Among the three main forms of autophagy, macroautophagy is the most widely studied and best characterized process. Macroautophagy, hereafter referred to as autophagy, is characterized by the formation of a cytosolic double-membrane vesicle, the autophagosome. During autophagy, cytoplasmic proteins, organelles or other materials are surrounded by phagophores, which expand and close to form autophagosomes. These autophagosomes fuse with lysosomes (or vacuoles) to form autolysosomes, in which the cytoplasmic cargos are degraded by resident hydrolases. The resulting degradation products are then transported back into the cytosol through the activity of membrane permeases for reuse [57]. Although autophagy is generally considered to be nonspecific, there are many examples of selective autophagy, including mitophagy (for mitochondria), ribophagy (for ribosomes), pexophagy (for peroxisomes), and reticulophagy (for the endoplasmic reticulum, ER) [58].

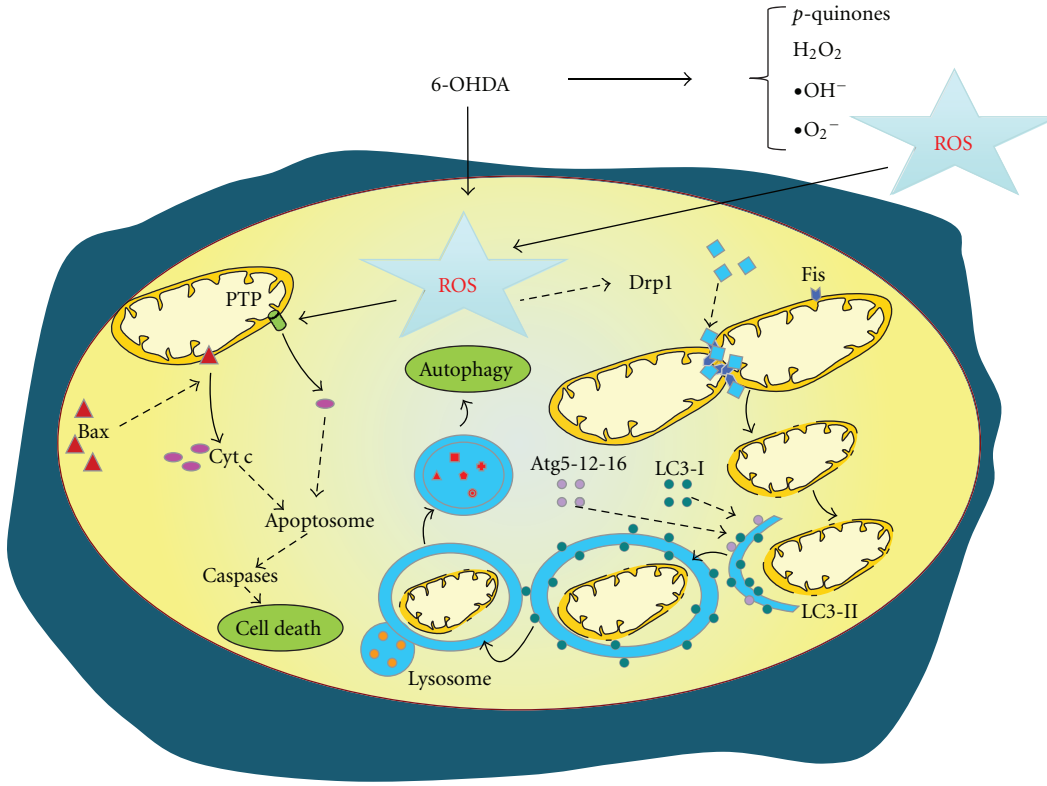

Figure 1: Mitochondrial fission and autophagy events are activated after 6-OHDA addition. Drp1 and Bax translocate from the cytosol to mitochondria. Drp1 has been proposed to encircle mitochondria to mediate constriction and this is followed by scission, which results in the formation of two separate mitochondria. Mitochondrial fission may play a role in the removal of dysfunctional mitochondria with reduced mitochondrial membrane potential, through an autophagy-lysosomal pathway named "mitophagy."

The primary role of autophagy is to protect cells under stress conditions such as starvation. During periods of starvation, autophagy degrades cytoplasmic materials to produce amino acids and fatty acids that can be used to synthesize new proteins or are oxidized by mitochondria to produce ATP for cell survival [59]. However, when autophagy is excessively induced, it can result in autophagic cell death, so-called type II programmed cell death [60, 61] (Figure 1). In addition to stress management, autophagy is involved in normal development [60], senescence [62], lifespan extension [63], immunity, and defense against microbial invasion [64]. In particular, autophagy has been observed to be deregulated in PD brains [65]. Consistent with these observations, suppression of basal autophagy causes neurodegeneration in mice [66]. Moreover, rapamycin, a well-known autophagic inducer, protects from PD toxins [67].

Unfortunately, to date it remains unknown what the underlying mechanisms of autophagy are in terms of procell survival versus procell death effects. Therefore, mechanisms that underlie these dual functions of autophagy (cell survival and cell death) need to be explored in the future. There are several hypotheses. The procell death effect of autophagy could be related to the activation of apoptosis, which would imply that autophagy is an upstream event of apoptosis [68]. For the cytoprotective effect of autophagy against stress, one possible mechanism for autophagic cell death could involve the autophagic degradation of a negative effector of apoptosis. This is supported by a recent demonstration that autophagic degradation of the *Drosophila* inhibitor of apoptosis (IAP) dBruce controls apoptotic cell death in nurse cells during late *Drosophila melanogaster* oogenesis [68]. Alternatively, autophagic degradation of active caspase-8, a positive effector of apoptosis, may also be responsible for the inhibition of apoptotic cell death in mammalian cells [69].

Autophagy is induced by 6-OHDA treatment. The 6-OHDA-induced autophagy correlated with an increase in the LC3-II protein level and with the accumulation of autophagic vacuoles in the cytoplasm and the activation of lysosomes [70]. It remains to be determined whether the induction of autophagy by 6-OHDA is related to cell death or to a cytoprotective response, which is activated by dying cells in order to cope with stress. In a previous study, tyrosine hydrolase-positive neurons in substantia nigra were protected from 6-OHDA-induced cell death when they were pretreated with the autophagy inhibitor 3-methyladenine [70]. On the other hand, experiments using neuron-specific knockout mouse models have demonstrated that autophagy deficiency leads

to protein aggregation and neurodegeneration, even in the absence of disease-related aggregate-prone proteins [58].

4. ROS as Second Messengers in 6-OHDA-Induced Pathways

Reactive oxygen species (ROS) are important for execution of physiological functions. However, excessive production of ROS is detrimental to the cell. Following an increase in ROS production, the cell's redox equilibrium is shifted to a more oxidized state, affecting both the structure and the function of different molecules. This may lead to specific toxic processes, which compromise the redox status of the cell and can cause cell death. Due to high levels of polyunsaturated fatty acids in their membranes and the relatively low activity of endogenous antioxidant enzymes, cells in the brain are particularly susceptible to oxidative damage.

On the other hand, ROS are able to induce pore opening [71]. Exposure of mitochondria to these species causes a decrease in the content of thiol residues in the membrane. It also leads to a collapse of the mitochondrial electrical transmembrane potential [72], which is prevented by the presence of antioxidant drugs like vitamin E and glutathione.

Under physiological conditions, 6-OHDA is rapidly and nonenzymatically oxidized by molecular oxygen to form 1,4-para-quinone and its degradation products [73], along with production of ROS such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical ($^\bullet OH^-$). Quinones react with nucleotic groups of macromolecules, leading to inactive or destroyed quinoproteins, which do not seem to contribute significantly to the observed cytotoxic effects of 6-OHDA. H_2O_2 can enter the cells and reacts with trace metals to form highly reactive $^\bullet OH^-$ [74]. This can oxidatively damage proteins, lipids, and DNA [75]. We have shown that 6-OHDA concentrations that were nontoxic to cell cultures did not significantly increase H_2O_2 production [76]. Moreover, H_2O_2 addition to cultures produced a pattern of cell death similar to 6-OHDA.

In addition to the non-enzymatic self-auto-oxidation process, microinjection of 6-OHDA into the striatum may lead to the generation of H_2O_2 via a mitochondrial enzymatic oxidation process. Inhibition of complex I of the electron transport chain also stimulated mitochondrial production of superoxide radicals. These superoxide radicals were then catalyzed to H_2O_2 by superoxide dismutase and, subsequently, $^\bullet OH^-$ may arise from the breakdown of H_2O_2. This may be associated with the mitochondrial dysfunction seen in our experiments, because $^\bullet OH^-$ rapidly attacks other biological molecules. The radicals produced in molecules such as lipids and proteins may also interact with mitochondrial enzymes to cause degradation.

In the signaling pathways that are involved in 6-OHDA-induced mitochondrial fission and autophagy, evidence revealed a key role for ROS. Our data demonstrated a relationship between ROS and 6-OHDA-induced mitochondrial fission and, subsequently, mitophagy. Intriguingly, 6-OHDA increases H_2O_2 between the cells. We made this observation using the dye CM-H_2DCFDA to measure peroxide-like formation. This specific tool allows us to ascertain the role of ROS in the mitochondrial dynamics process. Given that the inhibition of this dynamic process, using mdivi-1, did no block mitochondrial H_2O_2 production upon 6-OHDA treatments, H_2O_2 production is upstream of mitochondrial fission. In addition, TEMPOL and MnTBAP, two well-known antioxidant drugs, abolished translocation of Drp1 to mitochondria and, consequently, 6-OHDA-induced mitochondrial fission. In keeping with this interpretation, oxidative stress might be responsible for induced mitochondrial fission in several processes, including PD, perhaps due to a posttranslational redox change in the Drp1 protein [77, 78].

In addition, nitric oxide induces profound mitochondrial fission [44]. Cultured cerebrocortical neurons exposed to the physiological NO donor, S-nitrosocysteine, induced SNO-Drp1 formation and led to the accumulation of excessively fragmented mitochondria. SNO-Drp1-induced mitochondrial fragmentation caused synaptic damage, an early characteristic feature of AD and, subsequently, apoptotic neuronal cell death. Importantly, blockade of Drp1 nitrosylation (using the Drp1 (C644A) mutant) prevented A-β-mediated mitochondrial fission, synaptic loss, and neuronal cell death, suggesting that the posttranslational modification (S-nitrosylation) of Drp1 contributes to the pathogenesis of AD. Thus, SNO-Drp1 may represent a potential new therapeutic target for protecting neurons and their synapses in sporadic AD. Multiple groups have now reported on S-nitrosylation and subsequent activation of dynamin family members, including Drp1 [79–82].

In conclusion, although we await further clarifications on the role of mitochondrial fission and mitophagy in PD, we consider this pathway as a promising new and attractive pharmacological target. Interestingly, recent evidence has identified new molecules involved in PD such as Parkin and PINK1, key regulators of mitophagy.

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