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Calcium Homeostasis, Free Radical Formation, and Trophic Factor Dependence Mechanisms in Parkinson's Disease

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I. Introduction

PD† is a degenerative disorder of the central nervous system characterized clinically by akinesia, muscular rigidity, and tremor. The discovery that this disorder is directly caused by the degeneration of the nigrostriatal dopaminergic pathway is a major landmark relating a neurological disease to a specific neuronal system. In fact, the progressive loss in the SNc of dopaminergic neurons and their axons that project to the striatum results in an imbalance in the relative activity of striatopallidal and striatonigral output pathways, with the former becoming dominant. Increased striatopallidal activity consequently increases the output from the inhibitory neurons of the basal ganglia (Klockgether et al., 1991). In the early stages of the disease, most symptoms are remarkably improved by replacement therapy with L-dopa, which functions as an excessive substrate to maximize dopamine synthesis in the remaining dopaminergic neurons. However, the initially almost totally compensatory response to L-dopa therapy becomes insufficient with the progression of neuronal degeneration.

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† Abbreviations: PD, parkinson's disease; SN, substantia nigra; SNc, substantia nigra pars compacta; L-dopa, L-3,4-dihydroxyphenylalanine; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine·HCl; SNr, substantia nigra reticulata; mRNA, messenger ribonucleic acid; MPP^+, methylphenyltetrahydropyridinium ion; NMDA, N-methyl-D-aspartic acid; PC12, rat pheochromocytoma-12 cell line; DNA, deoxyribonucleic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid·HBr; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium (dihydropyridinium ion); ATP, adenosine triphosphate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline; TIQ, tetrahydroisoquinoline; mtDNA, mitochondrial deoxyribonucleic acid; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; GABA, γ-aminobutyric acid; GDNF, Glial cell line-derived neurotrophic factor.

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Within 5 years after the diagnosis of PD, the majority of patients receiving L-dopa exhibit fluctuations in their motor compensation that become less continuous and more difficult to manage (Shults, 1992).

Treatment strategies directed to halt the progressive loss of dopaminergic neurons in the midbrain are in development (Shults, 1992). These strategies focus on attempts to: (*a*) slow the progression of the pathogenic process; (*b*) protect remaining dopaminergic pathways that are still functioning; (*c*) increase the dopaminergic innervation of the striatum with neural transplantation in combination with neurotrophic factors; and (*d*) design drugs that inhibit events that limit the trophic factor efficacy.

Although the understanding of the pathophysiology of motor dysfunction in PD has improved, major progress in establishing an effective therapy is hampered by the inability to limit the progression of the pathological processes and by the fragmentary knowledge of the cause(s) leading to the selective damage of dopaminergic neurons. The question posed by such selectivity of neuronal loss is further compounded by the inability to define clearly the functionally decaying, as opposed to surviving, neuronal phenotypes. Thus, not all dopaminergic neuronal pathways are simultaneously affected; nor are all the dopaminergic neurons of the same group equally sensitive to the pathogenetic process (Gibb, 1992).

Possible causes for the loss of dopaminergic neurons in PD include an acute insult, chronic toxicity, and acceleration of the loss in dopaminergic neurons associated with normal aging. Clues to a pathophysiological mechanism that lead to selective dopaminergic cell loss can be obtained from at least four independent research trends: (*a*) comparison of the molecular mechanisms operative in the pathophysiology of the lesion in patients with PD with those occurring in dopaminergic neurons of animal models used to study the neurobiology of PD; (*b*) the viability of neurons in relationship to the destabilization of calcium homeostasis; (*c*) age-related changes in oxidative metabolism; and (*d*) investigation of specific factors prompting the survival of dopaminergic systems both in vivo and in vitro.

Recent reviews of PD (Jenner, 1992; Jenner et al., 1992; Javoy-Agid, 1992; Beal, 1992; Hirsch, 1992) focused on neurotoxicity due to free radicals of exogenous or of endogenous origin, with very little heeding to the indigenous susceptibility of the target neurons (other than looking into nonspecific deficits in the ability to deal with oxidative stress). However, if development of PD is triggered by abnormal formation of free radicals in the face of a toxic insult by either an exogenous product or an aberrant endogenous metabolite, the high specificity of the lesion still remains unexplained, unless it involves the formation of free radicals directly derived from dopamine. A phenomenon that deserves further study entails the presence of a latent genetic program in dopaminergic neurons that could trigger cell death in the presence of specific abnormalities in phenotypic protection of transmitter processing.

The purposes of this review are as follows: (*a*) to marshall supporting and opposing evidence for each of these viewpoints; (*b*) to evaluate the evidence for and against the generation of free radicals structurally related to dopaminergic transmission; (*c*) to propose a mechanism for the pathogenic process of cell death in PD; and (*d*) to look for a heuristic animal model that allows analysis of experimental and clinical evidence.

II. Neuropathology of the Parkinson's Disease Lesion

In the brains of patients who died with PD, not all dopaminergic cell groups were degenerated simultaneously to the same extent, indicating that neurons might not be equally resistant or susceptible to the intrinsic mechanism that triggers the disease (Kish et al., 1988; Gibb, 1992). The most severe neuronal loss occurs in the SNc, where the neurons that are most resistant to the intrinsic degenerative process usually bear Lewy bodies (which, however, are not pathognomonic of PD). Neuronal loss within the SN shows a preferential degenerative pattern of the lightly melanized dopamine-containing cells forming a ventral tier (or alpha strip) of SNc, in contrast to the heavily melanized cells forming the dorsal tier (or beta strip) of SNc that are relatively resistant to pathogenetic insult (Gibb, 1992).

This pattern is contrary to what one would expect from the tenets of the neuromelanin hypothesis that proposes a selective sensitivity to injury of melanin-rich dopaminergic neurons (Hirsch, 1992). In fact, according to Hirsch (1992), oxidative stress might be a cause for increased vulnerability of melanin-rich dopaminergic neurons. The apparent discrepancy for the proposed vulnerability of melanin-rich neurons in PD will be discussed in section IV.

An uneven pattern of neuronal loss was also reported for the nigrostriatal projections, with nearly complete deletion of dopaminergic markers in the caudal portions of the putamen; there is approximately 96% depletion in the dorsal rostral part of the caudate nucleus and about 60% reduction in the remaining parts of the caudate nucleus (Kish et al., 1988).

III. Animal Models of Parkinson's Disease

A. Methylphenyltetrahydropyridinium ion toxicity

The development of an irreversible PD-like syndrome in a group of meperidine abusers led to the discovery of the specific toxicology of MPTP in dopaminergic neurons (Davis et al., 1979; Langston et al., 1983). The differences between lesions in idiopathic PD and those caused in animals by MPTP are summarized in table 1. MPTP, which selectively damages cells in the SN (Burns

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TABLE 1

	Idiopathic Parkinson's disease	MPTP-induced disease
Course	Slowly progressive	Static or slow recovery observed after end of exposure to toxin
Pattern	1. Lesions extended beyond SN 2. Uneven pattern of cell loss within the SN	Largely restricted to SN Uneven pattern of cell loss (with chronic administration)
Neurochemistry	Greater depletion of dopamine in the putamen than in the caudate nucleus	Greater depletion of dopamine in the putamen than in the caudate nucleus (with chronic administration)
Manifestations	Rigidity, bradykinesia and resting tremor (3–5 Hz)	Rigidity and bradykinesia

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et al., 1983; Heikkila et al., 1984), is now used to obtain models for PD in monkeys and rodents. In the putamen of MPTP-treated monkeys, the [^{3}H]mazindol binding was substantially reduced, indicating a loss of neurons expressing the dopamine transporter. The characteristic topological pattern of dopamine depletion in the caudate nucleus observed in idiopathic PD, however, has not been found consistently in caudate nucleus of MPTP-treated monkeys (Moratalla et al., 1992).

Data acquired with histochemical techniques—in situ hybridization for dopamine receptor mRNAs, and neurotransmitter receptor-binding studies—made it possible to detect in neostriatum of monkeys two intermingled compartments called striosome and matrix. These compartments exhibit distinct afferent and efferent connections, with the striosomes receiving input from the prefrontal cortex, the insular cortex, the amygdala, and the matrix from association and sensorimotor cortex (Ragsdale and Graybiel, 1990).

The efferent projections of these two areas are also segregated, with striosomal neurons projecting mainly to the SNc and the majority of matrix neurons projecting to the pars externa and pars interna of the globus pallidus and to SNr. Dopamine D_1 recognition sites were shown to be diffusely distributed in matrix and striosomes, whereas muscarinic M_1-binding sites were denser in striosomes than in the matrix. In contrast, dopamine D_2 recognition sites, choline uptake, and benzodiazepine recognition sites were denser in the matrix than in striosomes (Graybiel, 1990).

When low doses of MPTP were injected in the caudate nucleus of monkeys, a selective vulnerability of matrix as compared with striosomes was observed (Moratalla et al., 1992). Interestingly, when a low MPTP-dose paradigm is used, within the MPTP-affected regions, the [^{3}H]mazindol binding in striosomes is relatively preserved as compared with the surrounding matrix (Moratalla et al., 1992). However, in idiopathic PD, a lesion pattern that selectively targets the matrix has not yet been described. MPP$^+$-induced toxicity in the rat is spontaneously reversible in most cases because only a transient loss of the expression of the tyrosine hydroxylase phenotype was reported and not an irreversible damage of dopaminergic neurons (Schneider, 1992; Schneider et al., 1992). Using Nissl staining, Beal et al. (1993) found a loss in total cell number in rat SNc, but the assessment of whether MPP$^+$ toxicity affects a specific cell phenotype, such as dopaminergic neurons expressing tyrosine hydroxylase, is still not definitive.

1. Protective effect of gangliosides. Another, yet poorly understood, part of the pathophysiology of MPTP is the mechanism by which the ganglioside, GM1, exerts its beneficial effects. GM1 was shown (*a*) to prevent MPTP-induced neurotoxicity in mice (Gupta et al., 1990) and (*b*) to promote recovery in MPTP-lesioned mice, rats, cats, and monkeys (Janson, 1992; Hadjiconstantinou et al., 1989; Schneider, 1992; Schneider et al., 1992).

Better understood is the mechanism by which gangliosides protect neurons against NMDA excitotoxicity. The protective action seems to occur downstream from the integral membrane receptor protein on a mechanism that governs the Ca^{2+} signaling amplification. Gangliosides spare the mechanism of signal transduction at the ionotropic and metabotropic glutamate receptors (Manev et al., 1990). De Erausquin and coworkers (1990) showed that in primary cultures of cerebellar granule cells, gangliosides protect neurons against NMDA-induced toxicity by preventing a deregulation of Ca^{2+} homeostasis. This important protective action of gangliosides is achieved by inhibiting protein kinase C activation and the translocation of the enzyme from the cytosol to the membrane elicited by persistent glutamate receptor stimulation (Favaron et al., 1990).

The mechanism whereby gangliosides accelerate the functional recovery of dopaminergic neurons is still unknown. Administration of GM1 to untreated mice had no apparent effect on dopaminergic functions. In MPTP-treated mice, GM1 significantly accelerated recovery in all age groups ranging from 8 weeks to 20 months, but in older mice, the recovery was delayed and was less complete than that in young mice. This effect seemed to depend upon the induction of compensatory mechanisms

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of dopamine synthesis and release from remaining terminals, possibly by facilitating the trophic action of yet unknown factors (Hadjiconstantinou and Neff, 1988; Schneider, 1992). This antineurotoxic and proneurotrophic action of GM1 was also observed in MPP^+-treated mesencephalic neurons in primary culture. GM1 restores the reduction of tyrosine hydroxylase immunoreactivity and dopamine uptake elicited by MPP^+ (Dalia et al., 1993).

The provisional hypothesis that was reached with MPTP lesion experiments maintains that gangliosides might increase the efficacy of a trophic factor (perhaps FGF) via a facilitatory action on tyrosine kinase activation.

Taken together, the two mechanisms of neurotoxic protection afforded by gangliosides seem (*a*) to stimulate the process of recovery from MPTP-caused degeneration and (*b*) to limit glutamate-induced excitotoxicity by curtailing the process of Ca^{2+} signal amplification. In the former mechanism, a facilitation of tyrosine kinase activation might be operative, whereas in the latter, an inhibition of membrane translocation of cytosolic Ca^{2+}-dependent protein kinases might be the prevailing molecular mechanism. In PC12 cells, gangliosides were found to prevent neuronal cell death in neurotrophic factor-deprived conditions by preventing apoptotic DNA fragmentation (Ferrari et al., 1993).

B. Excitatory Amino Acid Receptors in Dopaminergic Neurons of Substantia Nigra

Immunocytochemical studies revealed the presence of AMPA-preferring glutamate receptor subunits in the dopamine-containing neurons of rat SNc (Martin et al., 1993). Most of the dopaminergic neurons identified by tyrosine hydroxylase immunoreactivity were also immunoreactive to antibodies raised against GluR1-AMPA receptor subunits (Martin et al., 1993), whereas only some reacted to those against GluR2/3- and none against GluR4-specific epitopes (Petralia and Wenthold, 1992). Autoradiographic studies in the SNc of human brain revealed the presence of a low number of NMDA-binding sites (20 fmol/mg protein); the number was significantly reduced in patients with PD (2.6 fmol/mg protein) (Di Fazio et al., 1992). However, the binding affinity and the maximal binding capacity of [^{3}H]dizocilpine, a noncompetitive NMDA receptor antagonist, were not altered in caudate nucleus, putamen, temporal, or frontal cortex of parkinsonian brains (Holemans et al., 1991). The density of AMPA-binding sites in normal SNc is about ten times higher (175 fmol/mg protein) than that of NMDA, and is significantly reduced in PD (99 fmol/mg protein). In contrast, the density of metabotropic receptor sites is similar in SN of normal and PD patients (Di Fazio et al., 1992).

NMDA receptors in nigral dopaminergic neurons could not be detected by immunohistochemistry (Petralia et al., 1994). Data on the expression of mRNA encoding for various NMDA receptor subunits in dopaminergic neurons has not received the necessary focus, but the mRNA encoding for subunits of the NMDA receptor does not seem to be abundant. This might explain why NMDA fails to mediate excitotoxicity in primary cultures of embryonic mesencephalic neurons (de Erausquin, et al., 1994). Whole cell recording of dopaminergic neurons in slices of rat SN following afferent electrical stimulation showed that excitatory postsynaptic potentials have a prominent fast component induced by non-NMDA channel currents that participate in glutamate signal transduction followed by a smaller slow component contributed by the opening of NMDA channels (Mereu et al., 1991). On the other hand, injection of kainic acid (but not NMDA), into the SNc-induced seizures in rats: the seizures are prevented by kynurenic acid, an NMDA- and AMPA-receptor antagonist, when given in high doses (Maggio et al., 1990). Injection of AMPA into SNc prolongs rotatory behavior contralateral to the injection side (Masco, de Erausquin, Isaacs, Jacobowitz, Hanbauer and Gale, unpublished observation). This is in keeping with the view that nigral dopaminergic neurons express mainly non-NMDA selective glutamate receptors.

Contrary to these findings, NMDA was reported to induce [^{3}H]dopamine release from striatal slices (Bowyer et al., 1991) or from striatal synaptosomes (Araneda and Bustos, 1989; Llinas et al., 1984; Krebs et al., 1991), suggesting that NMDA receptors might be located on dopaminergic axonal terminals. An explanation for these seemingly contradictory data is offered by a report showing that stimulation of NMDA receptors located in postsynaptic neurons activates nitric oxide synthase. The newly formed nitric oxide might diffuse almost instantaneously to presynaptic dopamine nerve endings where it stimulates [^{3}H]dopamine release (Hanbauer et al., 1992).

C. Excitotoxicity in Dopaminergic Neurons: Dopaminergic and Glutamatergic Interaction

In rats, selective destruction of the striatonigral projection neurons reduced striatal NMDA-binding sites to a greater extent than those of AMPA, kainate, or glutamate metabotropic recognition sites (Tallaksen-Greene et al., 1992). The striatal matrix, which receives an input from neurons that are selectively affected by MPTP, has a greater number of binding sites for [^{3}H]AMPA and [^{3}H]dizocilpine than the striosomes, whereas the striosomes have a greater number of [^{3}H]kainate-binding sites than the matrix (Dure et al., 1992). A possible explanation for these findings could be that NMDA and AMPA receptors are present in different neurons but participate in the same circuit.

In spite of the weak and inconsistent evidence of a role of NMDA receptors in mediating dopaminergic cell death, several competitive NMDA receptor antagonists

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were reported to provide temporary protection against MPTP- and MPP^+-induced toxicity when they were either coadministered with the toxicant into the SNc, or injected systemically in rats (Klockgether and Turski, 1990; Turski et al., 1991). A similar protection by dizocilpine was observed in monkeys (Zuddas et al., 1992). The protection by these antagonists was only temporarily effective and might have been attributable either to the difference in the pharmacokinetics of the toxicant and the glutamate antagonists or to a potential radical scavenging action of the NMDA-receptor antagonists. However, other investigators failed to obtain protection in mice with NMDA antagonists against MPTP toxicity either acutely (Sonsalla et al., 1992; Kupsch et al., 1992) or after treatment protracted for 20 days (Kupsch et al., 1992). It was reported that in decorticated rats, which have a reduced excitatory input to the striatum, NMDA antagonists also decrease the MPP^+-induced lesions of striatal dopaminergic terminals and halt the loss of dopamine-containing neurons in the SNc (Beal et al., 1993). These data are incompatible with the theory that MPP^+-induced dopaminergic neuronal death might require activation of NMDA receptors. In line with this hypothesis are data showing that the protection by NMDA antagonists in decorticated rats might be caused by an extracortical excitatory input to the striatum that may be operative in the suppression of the dopaminergic phenotype by MPP^+ (Beal et al., 1993).

A second site for dopaminergic and glutamatergic interaction is the subthalamic nucleus, where glutamatergic output was increased after SNc destruction in various experimental mammals (Greenamyre and O'Brien, 1991). Overactivity of the subthalamic nucleus glutamatergic excitatory projections to the lateral globus pallidus and the SNr is postulated to play a central role in the clinical manifestations of PD. In MPTP-treated monkeys, the ablation of these glutamatergic projections reduces akinesia, rigidity, and tremor (Bergman et al., 1990; Greenamyre and O'Brien, 1991). Injection of NMDA receptor antagonists in the subthalamic nucleus, as well as in the lateral globus pallidus and SNr, reduces akinesia and rigidity in monoamine-depleted rats (Greenamyre and O'Brien, 1991) or in MPTP-pretreated monkeys (Graham et al., 1990). Interestingly, identical effects were described using NBQX, a specific antagonist of the AMPA-kainate-glutamate-receptor subtype (Klockgether et al., 1991), which also augmented the action of L-dopa. In another study, neither NBQX nor NMDA receptor antagonists were effective by themselves, but coadministered with L-dopa, they facilitated its antidystonic effects (Loschmann et al., 1991).

D. Effect of Excitatory Amino Acids on $[Ca^{2+}]i$ Homeostasis in Dopaminergic Neurons

A breakdown of the regulation of intracellular ionized calcium concentrations ($[Ca^{2+}]i$) leads to severe cell dysfunction and cell death (Siesjo and Bengtsson, 1989). Different portions of a neuron simultaneously carry a variety of functional processes that involve an increase of $[Ca^{2+}]i$ in spatial domains requiring a different extent of increase and specific time courses (de Erausquin et al., 1992; Miller, 1992; Alkon and Rasmussen, 1988; Carafoli, 1987).

The regulation of $[Ca^{2+}]i$ homeostasis depends on (*a*) specialized membrane segments that contain clusters of specific ionic channels, neurotransmitter receptors, calcium transporters, and pumps; (*b*) on subcellular organelles with calcium trapping/releasing functions within well-defined compartments (e.g., mitochondria in dendritic spines); and (*c*) on the presence of specific types of calcium-binding proteins. In theory, the mechanisms involved in the modulation of $[Ca^{2+}]i$ signals could vary not only in different neuronal phenotypes but also within the same type of neuron. If abnormalities in $[Ca^{2+}]i$ homeostasis are responsible for the selective neurodegeneration of dopaminergic neurons in PD, it seems essential to understand the regulation of $[Ca^{2+}]i$ in order to identify a signaling process involved in neuropathology.

1. Calcium homeostasis in cultured dopaminergic neurons. Information on the spatial distribution, within a neuron, of the different types of voltage-dependent calcium channels has been obtained by radioligand-binding measurements (Sanna et al., 1986), by channel-specific fluorescent ligands (Robitaille et al., 1990, Cohen et al., 1991), and by immunohistochemistry (Westenbroek et al., 1990). However, the specific functions associated with different channel subtypes are not understood. In dopaminergic neurons, measurements of $[Ca^{2+}]i$ steady state and Ca^{2+} fluxes made it possible to distinguish differences in the functional role of $[Ca^{2+}]i$ pools in soma and neuronal fibers. [^{3}H]Dopamine release from neurites of cultured mesencephalic neurons is consistently associated with the increase of neuritic $[Ca^{2+}]i$ that is abated by N-type channel blockers. This pool seems to generate the transient increase of $[Ca^{2+}]i$ in the vicinity of sites probably linked to the regulation of dopamine secretion (de Erausquin et al., 1992). Depolarization with K^+ induces a widespread increase of $[Ca^{2+}]i$ in the cell. N-type voltage-dependent calcium channel blockers attenuate the increase in $[Ca^{2+}]i$ in neurite and selectively prevent [^{3}H]dopamine release (de Erausquin et al., 1992). L-type voltage-dependent calcium channels, on the other hand, seem to be responsible for the increase of $[Ca^{2+}]i$ in the soma of dopaminergic neurons, but these channels could not be linked to the function of dopamine release, because L-type channel blockers failed to prevent [^{3}H] dopamine release (de Erausquin et al., 1992).

II primary cultures of embryonic rat ventral tegmental mesencephalon, NMDA (over a large concentration range) failed to increase significantly somatic $[Ca^{2+}]i$ in Mg^{2+}-free buffer. This result suggests that NMDA receptors either become desensitized very rapidly or are pres-

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ent in very low numbers (de Erausquin et al., 1994). In contrast, $[Ca^{2+}]i$ in dopaminergic neurons was increased selectively by stimulation of non-NMDA-type glutamate receptors and seemed to be the basis for the atypical vulnerability of dopaminergic neurons present in these cultures (de Erausquin et al., 1994). In fact, excessive stimulation with AMPA and other agonists of this receptor caused destabilization of the $[Ca^{2+}]i$ homeostasis, leading to atypical death of dopaminergic neurons (de Erausquin et al., 1994). In dopaminergic neurons, the increase of $[Ca^{2+}]i$ elicited by AMPA and agonists of the same receptor subtype also involves the activation of L-type, voltage-dependent calcium channels, because nifedipine was able to abate the increase of $[Ca^{2+}]i$ elicited by AMPA receptor agonists (table 2) (de Erausquin et al., 1994). The persistent destabilization of $[Ca^{2+}]i$ homeostasis elicited by AMPA agonists occurred exclusively in dopaminergic neurons, whereas in other neurons of the same culture dish, $[Ca^{2+}]i$ rapidly returned to normal levels (de Erausquin et al., 1994). In fact, 6 to 7 h after a 5-min exposure to 50 μM-AMPA, propidium iodide uptake was measurable only in dopaminergic neurons (de Erausquin et al., 1994). This phenomenon is compatible with the concept of destabilization of $[Ca^{2+}]i$ homeostasis elicited by abusive stimulation of glutamate receptors and suggests the possibility that therapeutic agents that act on targets downstream from the receptor (gangliosides?) may arrest the cascade of events leading to cell death (Guidotti et al., 1991).

In dopaminergic neurons, the persistent stimulation of the AMPA receptor also seemed to involve the dynamics of intracellular calcium stores, because pretreatment with the ryanodine receptor antagonist, dantrolene, reduced the increase of $[Ca^{2+}]i$ homeostasis (de Erausquin et al., 1991). Hence, the calcium-induced calcium release might play a role in the protracted elevation of $[Ca^{2+}]i$ following persistent stimulation of AMPA receptors. In central nervous system neurons, the demonstration of calcium-sensitive calcium stores has remained elusive; most neurons either do not express such a mechanism or do so under very particular circumstances, such as $[Ca^{2+}]i$ overload during membrane depolarization (Fohrman et al., 1993). In primary cultures of mesencephalic neurons, an overload of $[Ca^{2+}]i$ could also be caused by a reduction of either the content or the expression of calcium-binding proteins that have $[Ca^{2+}]i$ buffering capacity.

2. Calcium-binding proteins. The few calcium-binding proteins that have been studied in relation to neurodegeneration belong to a large family of more than 200 proteins structurally characterized by multiple repeats of the EF-hand motiv (Heizmann and Braun, 1992). (EF-hand is a structural motif in proteins, which binds Ca^{2+} selectively and with high affinity.) In the nervous system, the most abundant members of this protein family are parvalbumin, calbindin-D_{28K}, and calretinin (table 3). They are present in distinct subpopulations of neurons, providing an additional marker for specific calcium-dependent processes (Baimbridge et al., 1992). These highly soluble proteins are present throughout the cytosol, from soma to thin neurite terminals and seem to be useful for studies of neuronal connectivity and shape. Their function, however, remains largely unknown, and there is little information on specific properties of the neurons that contain them. The presence of parvalbumin and calbindin-D_{28K} in the dendritic trees of several types of neurons was linked to the excitability of these neurons (Baimbridge et al., 1992; Celio, 1986). Stable transfection of calbindin-D_{28K} into a cell line reduced the visualization of Ca^{2+} entry through voltage-dependent calcium channels and improved the ability of cells to reduce $[Ca^{2+}]i$ transients evoked by depolarization (Lledo et al., 1992). Another report, however, argued against a Ca^{2+}-buffering activity of calbindin-D_{28K}, because its Ca^{2+}-binding ability seems to be already maximal under resting conditions (Leathers et al., 1990).

Calretinin, a protein highly homologous with calbindin-D_{28K}, is present in subsets of neurons throughout the brain (Jacobowitz and Winsky, 1991). Colocalization of calretinin and tyrosine hydroxylase was found at E16 in SN_r and at E20 in SN_c. However, dopaminergic neurons in mesencephalic cultures from 14-day-old rat embryos do not possess calretinin immunoreactivity (Isaacs, Hanbauer and Jacobowitz, unpublished observations). So far, calretinin serves mainly as a neuronal marker, but its function remains completely obscure.

The initial clue for a role of $[Ca^{2+}]i$ homeostasis destabilization in the pathogenesis of PD was derived from data on calcium-binding proteins in postmortem brains of PD patients (Iacopino and Christakos, 1990). In MPTP-injected mice (Iacopino et al., 1992) and monkeys (German et al., 1992; Lavoie and Parent, 1991), calbindin-D_{28K}-immunoreactive/dopamine-containing cells are significantly spared. The content of calbindin-D_{28K} and the expression of its mRNA are not altered in MPTP-

TABLE 2
Effect of voltage-dependent Ca^{2+} channel antagonists on the AMPA-elicited increase in $[Ca^{2+}]_i$

Ca^{2+} channel antagonist	Type of channel	Effect on AMPA-elicited increase of $[Ca^{2+}]_i$
Nifedipine	L	Inhibition
ω-Conotoxin	N	None
Flunarizine	T	None

TABLE 3
Calcium-binding proteins in the brain

Present in most cell types	Present in neurons in central and peripheral nervous system
Calmodulin	Calretinin
Calcium-dependent proteases	Calbindin-D_{28K}
Protein kinase C	Parvalbumine
Actinin	$S100_{\alpha}$

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treated animals (Iacopino et al., 1992), which suggests that calbindin-D_{28K}-containing neurons are not lesioned by the exposure to the toxicant. In contrast, calbindin-D_{28K}-containing neurons were damaged in nucleus basalis of Meinert in brains of PD patients (Gibb, 1992). Calbindin-D_{28K}-specific mRNA and protein levels were decreased in SNc, hippocampus, and raphe dorsalis of patients with PD when compared with age- and sex-matched controls (Iacopino and Christakos, 1990), but other reports showed that calbindin-D_{28K} immunoreactive neurons were relatively spared in the SNc of PD patients (Yamada et al., 1990; German et al., 1992). Similarly, in primary cultures of rat embryonic mesencephalon, the calretinin-positive neurons are spared by excitotoxic injury caused by exposure to AMPA or kainate. In contrast, dopaminergic neurons in the same culture are irreversibly lesioned (Isaacs, Jacobowitz, de-Erausquin and Hanbauer, unpublished observation). Calretinin is found altered in genetically epilepsy-prone rats (Montpied, Winsky and Jacobowitz, personal communication). The question of whether calbindin-D_{28K} or calretinin may protect dopaminergic neurons from deregulation of $[Ca^{2+}]i$ homeostasis in PD is still unresolved.

3. $[Ca^{2+}]i$ homeostasis linked to mitochondrial function. Under normal circumstances, the impact of long-lasting $[Ca^{2+}]i$ signals in neurons is regulated by accumulation and release of Ca^{2+} from mitochondria (Denton and McCormack, 1990). The protein complex of the respiratory chain, located within the inner mitochondrial membrane, extrudes protons from the mitochondrial matrix, creating a mitochondrial membrane potential of 150–180 mV (negative inside). This gradient drives the synthesis of ATP and fuels the transport of Ca^{2+} inside the mitochondria.

Ca^{2+} is transported through the inner mitochondrial membrane by an electrophoretic uniporter without ion exchange, and it is extruded from the matrix in exchange for Na^+ (predominant mechanism in neurons) or protons. The increase into the micromolar range of $[Ca^{2+}]_i$ in cytosol and mitochondrial matrix results in saturation of the Na^+/Ca^{2+} exchanger. Under this condition, the rate of Ca^{2+} efflux from the matrix will be constant, whereas the rate of Ca^{2+} influx from the cytosol remains fast. Mitochondrial Ca^{2+} accumulation will continue until cytosolic $[Ca^{2+}]i$ has decreased to a level at which the transport across the inner membrane is at steady state. This point is known as the mitochondrial "set point" (Nicholls, 1985). If cytosolic $[Ca^{2+}]i$ decreases below this point, the net transport will turn outward, resulting in prolonged Ca^{2+} release from the matrix and slowing the decline of $[Ca^{2+}]i$ (Miller, 1992). Abnormal mitochondrial function could conceivably cause a shift of the "set point." For example, a large Na^+ influx evoked by even mild increases in excitatory amino acid receptor stimulation (much below the level of stimulation usually used in the in vitro paradigm) could shift the "set point" at which mitochondria would maintain cytosolic $[Ca^{2+}]i$ at a higher level.

Increased vulnerability of neurons to $[Ca^{2+}]i$ overload could also arise from mitochondrial functional abnormalities in PD (Beal et al., 1993; see also section IV.E) that might be indirectly linked to deficient cytosolic content of calcium-binding proteins. It is possible that a reduced buffering capacity caused by diminished expression of calcium-binding proteins could make dopaminergic neurons even more vulnerable to excitotoxic injury, but experimental proof for such an allegation is still missing.

IV. Free Radical Formation: Exogenous Toxicants versus Abnormal Metabolism

A. Epidemiology

The discovery that MPTP causes a parkinsonian syndrome (Davis et al., 1979; Langston et al., 1983) established a working hypothesis that relates the etiology of PD to an environmental toxic insult. MPTP is not a naturally occurring compound and only accidentally caused a few cases of PD. However, when the route of MPTP metabolism by monoamino oxidase B in brain was understood, it became an interesting model to study the mechanism responsible for generating toxic metabolites (Jenner et al., 1992). Table 4 categorizes neurotoxicants into "exogenously formed" and "endogenously formed."

Although epidemiological evidence suggests that an environmental toxicant might not be a significant factor in causing the disease (Jenner, 1992; Jenner et al., 1992; Spencer et al., 1992), the possibility of risks associated with industrial or agricultural chemical contaminants cannot be excluded (Jenner et al., 1992).

On the other hand, the observation that 10% of patients with PD have an affected relative suggests the existence of a genetic component (Jenner, 1992). Studies of 65 pairs of twins seemed to exclude a major DNA contribution (Ward et al., 1983), but a more stringent study showed that the incidence of the disease in families of monozygotic and dizygotic twins was higher than expected from population prevalence rates (Vieregge et al., 1992). A familial form of PD inherited as an autosomal dominant trait with very low penetrance has been described (Jenner et al., 1992). Notwithstanding these findings, none of the described studies showed a major genetic impact in the etiology of PD and only indicated

TABLE 4
Exogenous and endogenously formed toxicants acting on dopaminergic neurons

Exogenous toxicant and metabolites	Endogenous toxicant
MPTP, MPP^+, $MPDP^+$	Dopamine metabolites
Cycasine, methylazomethanol	Tetrahydro-isoquinolines
β-N-methylamine-L-alanine	β Carbolines

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the presence of a genetic predisposition. It has been suggested that this inherited trait could be the inability to handle relevant toxicants (Jenner et al., 1992), but the evidence supporting this possibility is still inconclusive.

B. Exogenous Toxicants Acting on Dopaminergic Neurons

Selective toxicity in humans and nonhuman primates of MPTP toward dopamine-containing neurons of the SN initiated research on the possible neurotoxic origin of PD. MPTP is metabolized by monoamino oxidase B to produce $MPDP^+$, which in turn is converted to the pyridinium, MPP^+. This species is then transported by the dopamine transporter into nerve terminals and accumulated within mitochondria; there, it inhibits complex I of the mitochondrial respiratory chain (Singer and Ramsay, 1990), causes depletion of ATP (Jenner, 1992; Jenner et al., 1992), alters $[Ca^{2+}]i$ homeostasis (Wilson et al., 1991; Chiueh et al., 1993), and triggers cell death. In addition, the metabolism of MPTP to $MPDP^+$ and MPP^+ by monoamino oxidase B might produce superoxide radicals (Rosetti et al., 1988). Thus, the MPTP model prompted an increase in research directed toward unraveling the molecular mechanism that causes dopaminergic cell death. This trend led to a heuristic hypothesis useful in studying biochemical targets in postmortem human brain tissue. However, several unresolved differences still remain between MPTP-induced lesions and idiopathic PD. These include differences in the anatomical topology of neuronal death (Jenner et al., 1992) and in the specific patterns of the neurodegeneration (Spencer et al., 1992).

Although exposure to MPTP and other excitotoxic agents triggers distinct anatomical patterns of neuronal degeneration in humans and animals, these patterns diverge from those characteristic of PD (Jenner et al., 1992). In contrast to idiopathic PD, neuronal degenerations in the SN elicited by excitotoxicants or MPTP fail to progress once the toxicant has been metabolically removed from the tissue (Spencer et al., 1992). However, this does not rule out the possibility that some cases of PD might be etiologically linked to an environmental source that continuously generates a toxic factor selective for dopaminergic neurons.

Several reports in the literature suggest a role for food-contained excitatory amino acids in the etiology of PD. Methylazoxymethanol-β-glucoside (also known as cycasin) and the amino acid β-N-methylamino-L-alanine, both present in seeds of the cycad plant, might be involved in the pathogenesis of a parkinsonian syndrome also referred to as a progressive form of amyotrophic lateral sclerosis/PD/dementia complex (Spencer et al., 1987). Because this syndrome has until now been found only in a subpopulation of Western Pacific Islanders, it is undoubtedly caused by an environmental factor (Spencer et al., 1992).

β-N-Methylamino-L-alanine administration in monkeys elicits an acute syndrome reminiscent of amyotrophic lateral sclerosis/PD/dementia complex. After β-N-methylamino-L-alanine is taken up by neurons, it inflicts latent but nonprogressive neurotoxicity (Spencer et al., 1992). Interestingly, in neural tissue, cycasin is taken up through the glucose transporter and is then metabolized to methylazoxymethanol, a powerful neurotoxicant with DNA-alkylating properties. Alkylation of guanine results in the formation of 7-methylguanine (Spencer et al., 1992), which might act as a gene suppressor leading to metastatic transformation in mitotic cells and to abnormal gene expression and possibly neural degeneration in postmitotic cells (Spencer et al., 1992). In fact, another alkylating agent with neurotoxic properties, vincristine, induces apoptosis in hypothalamic neurons by a yet unknown mechanism (Muzylak and Maslinska, 1992). It is interesting to speculate that alkylation of guanine could facilitate the expression of programmed cell death through a permanent change in the methylation status of regulatory nucleotides.

C. Endogenous Toxicants Acting on Dopaminergic Neurons

Is it then possible that an endogenous toxicant that selectively destroys dopamine-containing cells is produced as a result of aberrant metabolism? So far, two possible groups of compounds have been studied in this respect: dopamine metabolites and condensation products, such as TIQ and β-carboline.

Dopamine metabolism by monoamino oxidase B, as well as dopamine auto-oxidation to neuromelanin, generate free radicals (semiquinones) and reactive oxygen species that might induce oxidative stress (Jenner et al., 1992). The evidence in favor of a role for oxidative stress is reviewed in section IV.D..

Methylated derivatives of TIQ have been identified in both normal and parkinsonian brains (the level being much higher in the latter) (Niwa et al., 1991) and in cerebrospinal fluid of parkinsonian patients (Moser and Kompf, 1992). In addition, a N-methyltransferase present in human brain catalyses N-methylation of TIQ using S-adenosyl-methionine as donor (Naoi et al., 1989). The naturally occurring dihydroxytetrahydroisoquinoline, salsolinol, is synthetized by a cytochrome P450 enzyme and can be methylated as described above (Maruyama et al., 1992; Suzuki et al., 1989). Interestingly, the rate of N-methylation of salsolinol is significantly higher in the SN than in other brain regions (Maruyama et al., 1992). Finally, in the SN of mice and marmosets, TIQ decreases tyrosine hydroxylase immunoreactivity without changing the cell number (Ogawa et al., 1989; Nagatsu and Yoshida, 1988).

β-Carbolines that contain the MPTP moiety inhibit mitochondrial respiration and cause cell loss in the SN

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of rats but do not cause cell loss, dopamine depletion, or parkinsonian symptoms in monkeys (Jenner et al., 1992).

D. Intrinsic Abnormalities in Oxidative Metabolism

A large body of literature suggests that oxidative metabolism plays a role in dopaminergic cell death in PD (Hirsch, 1992; Olanow, 1992; Jenner, 1992; Jenner et al., 1992; Youdim et al., 1993; Chiueh et al., 1993). The most salient finding that supports the hypothesis relating PD to oxidative stress is that in the SN of PD patients, the content of reduced glutathione is decreased, whereas glutathione peroxidase and catalase activity are not altered (Perry and Yong, 1986; Sofic et al., 1992; Jenner, 1992).

Increased lipid peroxidation has been detected at autopsy in the SN of PD patients. The SN of these patients also contains increased levels of malondialdehyde and decreased concentrations of total polyunsaturated fatty acids (Dexter et al., 1989). It is surprising that glutathione peroxidase, catalase, vitamin C, and vitamin E, although present in high concentrations in the SN, failed to prevent the increased formation of reactive oxygen species (Jenner, 1992).

The increase of mitochondrial superoxide dismutase activity and of the antioxidant ion, Zn^{2+}, might reflect a possible compensatory response to an increase of superoxide radical production in the SN of PD patients (Jenner, 1992; Jenner et al., 1992). This notion is further supported by the reduced levels of Cu/Zn superoxide dismutase mRNA in the SN of PD patients (Hirsch, 1992) and by the resistance to MPTP-induced toxicity of transgenic mice with increased expression of Cu/Zn superoxide dismutase (Przedborski et al., 1992).

Immunohistochemically, glutathione peroxidase was only demonstrable in glial cells of human SNc. In the SNc of parkinsonian patients, it was observed that a large number of glutathione peroxidase-containing glial cells surround the surviving dopaminergic neurons and that their number correlates with the severity of the dopamine cell loss (Damier et al., 1993). From this finding, one can infer that gliosis might be an attempt of the tissue to limit the superoxide radical-caused dopamine neuron death by increasing the number of glutathione peroxidase-containing cells in the SN of PD patients.

The hypothesis that neuronal damage in PD might involve the action of oxygen radicals also arises from studies on iron metabolism. Accumulation of nonchelated iron and a decrease in ferritin content in SN of patients with PD were found during later stages of the disease (Riederer et al., 1989; Jenner, 1992). However, in earlier stages of PD, neither the chelated nor free iron content of the SN differed significantly from that in controls. In line with these findings are data showing that death of dopamine neurons elicited by 6-hydroxydopamine is linked to a release of iron from its binding sites in ferritin. Coadministration of iron chelators could prevent the toxic effect of 6-hydroxydopamine in rats (Youdim et al., 1993).

Although the evidence of oxidative stress in the SN of patients with PD at the time of death might be convincing, it remains debatable whether this stress is a primary process or the consequence of other events. As pointed out by Jenner et al. (1992), even if formation of reactive oxygen species is not the primary process, its initiation at any stage during the course of the disease could cause devastating degeneration of dopaminergic cells in the SN and could precipitate the onset of PD symptoms. At this point, a commonly accepted view proposes that the degenerative process is slow and gradual and causes degeneration of dopaminergic neurons that is associated with senility (Jenner et al., 1992). In fact, the expression of mRNA encoding for the dopamine transporter in normal human brain declines as much as 40% in the sixth decade of life (Bannon et al., 1992), which indicates that during aging, dopaminergic cells undergo a significant spontaneous decrease in number, possibly caused by apoptosis. Thus, apoptosis, together with other toxic effects, might cause the loss of dopaminergic neurons and the decrease of their function. These data provide new support for the "double hit" hypothesis, which suggests that PD is the result of the combined effects of aging and toxic insult (Calne and Langston, 1983).

E. Mitochondrial Dysfunction

The discovery that mitochondria play a role in MPTP metabolism led to the initiation of research on mitochondrial function in patients with PD. Studies on the four respiratory chain complexes embedded in the inner mitochondrial membrane were carried out in postmortem brain tissue and show that the activity of β-nicotin-amide adenine dinucleotide (reduced form) coenzyme Q1 (complex I) was selectively reduced in the SN of patients with PD, whereas the other respiratory chain complexes (complex II, III, IV) were unaffected (Jenner et al., 1992). Hattori and coworkers (1991) confirmed these data by immunostaining experiments in the SN in eight PD patients, but they also found a reduction of succinate-coenzyme Q reductase (complex II) in three of these eight patients.

A deficiency in complex I was also observed in muscle and platelets of PD patients (Bindoff at al., 1989; Parker et al., 1989). Alterations in three specific subunits of the mitochondrial complex I were also reported in the striatum of PD patients, suggesting that a generalized deficit of mitochondrial complex I enzymes might be extended to other tissues (Mizuno et al., 1989). The nature of this deficit in mitochondrial enzymes reported in the SN needs further study; also, the nonspecific peripheral mitochondrial enzyme deficit must be investigated more extensively and carefully.

Several groups have attempted to clarify the molecular basis for the complex I defect. Complex I enzymes consist

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of seven polypeptides encoded by mtDNA and almost 20 other polypeptides (including iron-sulphur proteins) encoded by nuclear DNA. No differences were found in the polypeptide structure of the iron-sulphur proteins of normal and PD patients (Jenner et al., 1992).

Studies of restriction fragment length polymorphism of the mitochondrial genes encoding complex I enzymes showed no major deletions in spite of a deficient oxidative phosphorylation (Shoffner et al., 1991; Lestienne et al., 1990; Schapira et al., 1990; Nakagawa-Hattori et al., 1992). Ikebe et al. (1990) reported the presence of the so called "common deletion" in corpus striatum of parkinsonian brains. This deletion involved genes encoding four polypeptides of complex I ($mtDNA^{4977}$), and was detected in PD patients with progressive external ophthalmoplegia (Shoffner et al., 1989) and with mitochondrial myopathy (Holt et al., 1988). However, this deletion was also present in corpus striatum, cerebral cortex (Ikebe et al., 1990), and in platelets (Sandy et al., 1993) of age-matched controls. In fact, a recent study showed that in normal adults, an age-related increase of $mtDNA^{4977}$ deletion was detectable in caudate nucleus, putamen, and SN (Soong et al., 1992). These three regions are characterized by high rates of dopamine metabolism catalyzed by mitochondrial monoamino oxidase that results in the production of oxygen radicals. The progressive accumulation of this defect with aging could precipitate or amplify an underlying pathological process that could cause dopaminergic cell death (Beal et al., 1993). Point mutations in mtDNA have also been reported in patients with Alzheimer's disease, but the relevance of this finding remains to be established (Beal et al., 1993).

The mechanism whereby defects in mtDNA might lead to neuronal death is still unknown. The mitochondria-encoded polypeptides of complex I participate in the formation of an hydrophobic ring or shell around the iron-sulphur proteins, whose integrity seems necessary for enzymatic activity (Beal et al., 1993). Indeed, rotenone and pethidine (two powerful complex I inhibitors) bind to one of the mtDNA-encoded peptides, a site that reportedly also binds MPP^+ (Beal et al., 1993). Decreased complex I activity measured in SN of parkinsonian patients could lead to excessive production of superoxide radicals, which, in the presence of increased Fe^{3+} content, could promote production of highly destructive hydroxyl radicals (Beal et al., 1993). Interestingly, irrespective of the initial cause of the decrease of complex I activity (i.e., endogenous or exogenous pyridinium-like molecules versus a genetic defect), increased oxidative metabolism will lead to increased mutations of mtDNA and presumably to further damage of complex I (Soong et al., 1992). Such a positive feedback loop could lead to a catastrophic response, with a precipitous decline in the number of dopaminergic neurons at a time when the age-dependent decrease of dopaminergic functions occurs (Jenner et al., 1992; Bannon et al., 1992). Such a multifaceted mechanism could account for the delayed onset and progressive course of the disease (Beal et al., 1993).

V. Evidence of Neurotrophic Factor Dependency

A. Evidence from Transplant Experiments

The development and maintenance of the mammalian nervous system requires neurotrophic factors. Degeneration of nigrostriatal dopaminergic neurons might be partly attributed to reduced neurotrophic support. Neurotrophic factors enhance the survival of transplants of fetal dopaminergic cells or adrenal medulla into the striatum of animals by promoting the survival and neurite elongation of dopaminergic neurons (Shults, 1992).

In vitro, NGF facilitates the survival of transplanted neonatal rat adrenal medullary cells and promotes neurite elongation (Unsicker et al., 1983). In rats with a 6-hydroxydopamine-lesioned SN, NGF applied onto adrenal medullary grafts increases the number of surviving cells (Stromberg et al., 1985). A similar effect was obtained by cotransplanting C6 glioma cells with adrenal medullary cells (Westermann et al., 1988). Direct evidence of a regenerative response of neurotrophins in transplanted fetal dopaminergic cells is still lacking. In MPTP-treated monkeys with implantations of fetal SN, newly-sprouted collateral axons of dopaminergic neurons were found to reinnervate the corpus striatum. But transplantation of neonatal adrenal medulla, fetal cerebellum, or fetal spinal cord into lesioned rat corpus striatum or even a simple wound itself increases sprouting of dopaminergic axons and suggests that the beneficial effect of these grafts might be related to their ability to stimulate sprouting caused by the secretion of neurotrophic factors (Shults, 1992). A feature common to any of the above-described manipulations is the presence of inflammatory cells, microglia, and reactive astrocytes at the site of the manipulation. In fact, even transplantation of activated leukocytes into the striatum increases explant reinnervation and behavioral improvement (Weber et al., 1989).

B. Evidence from Tissue Culture and in Vivo Experiments

Only certain trophic factors can promote survival of dopaminergic neurons in vitro and enhance recovery of dopaminergic neurons after acute injury. Among the members of the neurotrophin family, only BDNF showed effects on growth and differentiation of dopaminergic neurons in vitro (Hyman et al., 1991, Knusel et al., 1991). The effect of BDNF was dose-dependent, increasing dopaminergic cell survival by three- to five-fold (Hyman et al., 1991), and protected cultured dopaminergic neurons from the toxic effect of 6-hydroxydopamine and MPP^+ (Spina et al., 1992). In vivo, however, the neuroprotective effect of BDNF has not yet been documented. When recombinant BDNF was administered directly at the medial forebrain bundle after ascending axons were severed from the ventral mesencephalon, it failed to

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initiate outgrowth of dopaminergic neurites (Knusel et al., 1992). In corpus striatum of adult mice only a minimal amount of BDNF mRNA is detectable by northern blot analysis (Hofer et al., 1990), whereas hippocampus and cerebellum, two structures virtually devoid of dopaminergic innervation, had the highest BDNF mRNA expression (Shults, 1992). Studies with a dopaminergic neuroblastoma cell line (SH-SY5Y) showed that BDNF, by inducing glutathione reductase, might protect against oxidative stress and 6-hydroxydopamine-elicited toxicity (Spina et al., 1992).

Insulin-like growth factor-1 and insulin itself, both present in the brain, stimulate dopaminergic cell development in vitro, but there is no evidence of a neuroprotective action in vivo (Fuxe and Agnati, 1992).

Basic and acidic FGF family act as trophic factors on dopaminergic neurons (Shults, 1992; Gaul and Lubbert, 1992). Both growth factors increased survival and promoted neurite outgrowth in mesencephalic dopaminergic neurons cultured in chemically defined media, and basic FGF also promoted survival of dopaminergic mesencephalic neurons in vivo (Otto and Unsicker, 1992). Wanaka and coworkers (1990) showed the presence of FGF receptor mRNA in the brain but found moderate expression in SN and only a small mRNA signal in the striatum. The effect of FGF (either acid or basic) on survival of cultured mesencephalic cells was found to be mediated by glial cell proliferation (Engele and Bohn, 1991). Indeed, inhibition of glia proliferation abolished the survival of dopaminergic neurons, whereas addition of conditioned media from mesencephalic glial cultures increased survival (Engele and Bohn, 1991).

In a detailed study, Beck and coworkers (1993) showed that BDNF stimulated survival, primary-neurite formation, and dopamine uptake. In contrast, insulin-like growth factor-1 promoted survival but stimulated neurites and dopamine uptake less effectively than did BDNF. Basic FGF increased both mRNA levels and tyrosine hydroxylase activity, but it increased only slightly the survival of dopaminergic neurons and dopamine uptake (Beck et al., 1993).

The ciliary neurotrophic factor and dopamine itself both lack survival-promoting properties, but they increase tyrosine hydroxylase immunoreactivity in mesencephalic cultures (Magal et al., 1993). The effect of dopamine was mediated by a dopamine D_2 receptor, and the effect of ciliary neurotrophic factor was independent of the presence of glial cells, suggesting its direct action on neurons (Magal et al., 1993).

A common denominator of all of these trophic factors is their lack of specificity. Insulin-like growth factor-1, acidic and basic FGF, and BDNF increase GABA or serotonin uptake (in addition to their effects on dopaminergic neurons) and are potent glial mitogens in midbrain cultures (Weiss, 1993).

Recently, a novel specific dopaminergic neurotrophic factor was isolated from a rat glial cell line, cloned, and characterized (Lin et al., 1993). GDNF is a glycosylated disulfide-bonded homodimer distantly related to the transforming growth factor-β-superfamily (Lin et al., 1993) and has many features in common with sulfated glycoprotein-2, a protein linked to neurodegeneration (May and Finch, 1992). In embryonic cultures, GDNF promoted survival as well as morphological and biochemical differentiation of dopaminergic neurons without affecting the growth of glial cells (Lin et al., 1993). Hence, it is possible that the effects of basic FGF, which depend upon glial cell proliferation, might be mediated through an enhanced secretion of GDNF.

When embryonic mesencephalic cells were cultured in the presence of extracts prepared from striata of haloperidol-treated adult rats (Carvey et al., 1989) or from brains of patients with PD (Carvey et al., 1993), both the survival and differentiation of dopaminergic neurons were promoted. Interestingly, striatal extracts of patients with PD contained more growth-promoting activity than did that of normal age-matched subjects, suggesting that a lack of trophic factor production might not have caused the disease (Carvey et al., 1993).

An alternative explanation for this phenomenon could be that increased secretion in the striatum of patients is a tardive compensatory response by the target cells (either neuron or glia). Yet another possible explanation is the loss of response to the growth-promoting activity in the adult mesencephalon due to a change in expression or regulation of receptors for neurotrophins. Inasmuch as the active principle in the extract has not yet been identified, it is difficult to establish the origin and the physiological importance of this growth-promoting factor.

It is tempting to speculate that the above described striatal extracts might contain GDNF, particularly in the light of the structural similarities it shares with glycoprotein-2. In fact, both GNDF and glycoprotein-2 are dimers formed by disulfide bonds, with a molecular weight between 32 to 42 and 34 to 47 kDa, respectively. In both cases each subunit has a cluster of cysteine residues (seven for GNDF, five for glycoprotein-2) and two to four asparagine-linked glycosylation sites (Lin et al., 1993; May and Finch, 1992). Glycoprotein-2 and similar molecules were isolated from several mammalian species and are associated with the responses of brain and other tissues to injury. Recently, a clone isolated from a human hippocampal cDNA library (pADHC-9) was shown to encode the human isoforms resembling rat glycoprotein-2 (May and Finch, 1992), which were increased in hippocampal tissue of patients with Alzheimer's disease. The same authors showed that perforans neuronal fiber path transection or kainate-induced excitotoxic lesions of the hippocampus also elevated glial and neuronal levels of glycoprotein-2 protein and its mRNA (May and Finch, 1992). In addition, in prostate

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epithelial cells, mRNA encoding glycoprotein-2 was found to be increased during programmed cell death induced by castration (May and Finch, 1992).

VI. α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid·HBr Receptor Abuse-Induced Apoptosis: A Model for Dopaminergic Cell Death in Parkinson's Disease

In several reviews, it was suggested that in neurons, an apoptotic program might be initiated by a set of factors (Altman, 1992; Lee et al., 1993). Apoptosis was shown to be a $[Ca^{2+}]i$-dependent process that requires new gene expression and protein synthesis. In nematodes, one of the two known apoptosis genes, (*ced-4*), expresses two putative calcium-binding domains (Altman, 1992).

As discussed before, the etiology of PD is characterized by dopaminergic cell loss that progresses slowly and by acute cell death that is induced by exogenous toxins. In different types of tissue, stimulation of certain subtypes of glutamate receptors might lead to an atypical vulnerability of specific neuronal groups, leaving others unaffected. These observations suggest that conditions such as deprivation of neurotrophins or stimulation of specific types of glutamate receptors might inappropriately activate programmed cell death. In fact, cell death occurs in the NGF-deprived rat PC12 cell line and in sympathetic neurons. In both cell types, apoptosis, triggered by lack of NGF, was prevented by transcription or translation inhibitors (Mesner et al., 1992).

Growth and maintenance of dopaminergic cells seem to depend on trophic factors. However, adult dopaminergic neurons seem to be relatively deprived of BDNF. Interestingly, the distribution of cell death in PD and in MPTP toxicity overlaps with the distribution of AMPA receptors. It was shown that exposure of primary cultures of cerebellar granule cells to MPP^+ stimulated nucleosomal DNA degradation and led to the induction of apoptosis (Dipasquale et al., 1991). Although it was demonstrated that in neurons, MPP^+ toxicity is linked to alterations of complex I mitochondrial enzymes, no experimental evidence could link alterations of mitochondrial function to apoptosis. Interestingly, overexpression of the mammalian proto-oncogene *bcl-2*, a membrane-bound protein that is associated with the nuclear envelope, the endoplasmic reticulum, and the inner mitochondrial membrane (Jacobson et al., 1993) prevents apoptosis in sympathetic neurons (Garcia et al., 1992) as well as in a neuronal PC12 cell line (Mah et al., 1993). However, in human mutant cell lines, which lack mitochondrial DNA and therefore fail to express a functional respiratory chain, apoptosis can still be induced, and overexpression of *bcl-2* still protects the cells from programmed cell death. These findings suggest that neither apoptosis nor the protective effect of *bcl-2* depend on mitochondrial respiration (Jacobson et al., 1993).

Spina et al. (1992) speculated that increased oxidative stress could trigger a faster-than-normal rate of senescence in dopaminergic neurons. The protective action of BDNF against 6-hydroxydopamine toxicity in a human dopaminergic neuroblastoma cell line was associated with the induction of glutathione reductase, an enzyme that prevents oxidative stress-induced damage in neurons. In rat SN, the 6-hydroxydopamine-elicited lesions of dopaminergic neurons were accompanied by a substantial increase of *c-jun* (but not *c-fos*) proto-oncogene expression (Jenkins et al., 1993). Inasmuch as the expression of *c-jun* and *c-fos* proto-oncogenes are also rapidly induced in cell lines undergoing apoptosis (Lee et al., 1993), it will be important to investigate whether the increased expression of these oncogenes might occur as a signal for neuronal regeneration or might initiate programmed cell death.

It has been suggested that minimal overstimulation of AMPA receptors might activate cell death by a mechanism involving destabilization of $[Ca^{2+}]i$ homeostasis. Cells low in calbindin-D_{28K} are more sensitive to excitotoxicity. Moreover, in prostate cell lines, the expression of a hybrid calbindin gene is specifically associated with protection against apoptosis in the absence of hormone (Lee et al., 1993), and overexpression of calbindin-D_{28K} protects lymphocytes against glucocorticoid-, cyclic adenosine monophosphate-, and calcium ionophore-induced apoptosis (Lee et al., 1993). Protection against apoptosis has also been demonstrated in PC12 neuronal lineages that have the characteristic of a dopaminergic phenotype. In this cell line, NGF was neuroprotective and induced the expression of two proteins related to S100 calcium-binding protein (Altman, 1992).

Interestingly, vincristine, a chemotherapeutic agent with remarkable neurotoxic side effects, induces apoptosis in neurons (but not in glial cells) in regions of the central nervous system where the blood-brain barrier is lacking (Muzylak and Maslinka, 1992).

The information discussed here is summarized in a unifying model for the mechanism of dopaminergic neuronal death (fig. 1). Based on the interaction of neurotrophic and neurotoxic factors with dopaminergic neurons, this heuristic model was designed with the aim to be useful in the search for new neuroprotective agents as well as new therapeutic approaches. From a pathological standpoint, various factors could trigger an aberrant cellular response that might damage dopaminergic neurons. However, a common feature of the discussed metabolic abnormalities is that they are accompanied with a loss of $[Ca^{2+}]i$ homeostasis that is consistently linked to dopaminergic cell death. For example, in the course of their normal activity, dopaminergic neurons continuously form free radicals that, during a lifetime, could lead to the formation of high levels of specific neurotoxicants or to the accumulation of mutated mitochondrial DNA. Mutations above a certain threshold could result in com-

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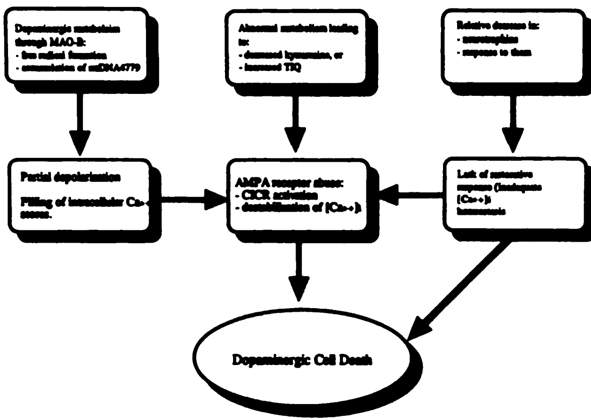

FIG. 1. Unifying model for the mechanism of dopaminergic neuronal death. MAO-B, monoamino oxidase-B; CICR, calcium-induced calcium release.

plex I dysfunction. In susceptible individuals, i.e., those with deficits in the production of kynurenic acid, minimal overstimulation of AMPA receptors could activate programmed cell death by a mechanism involving destabilization of $[Ca^{2+}]i$ homeostasis. Mitochondrial dysfunction, in this context, could produce partial depolarization and thereby facilitate the filling of the intracellular Ca^{2+} stores sensitive to $[Ca^{2+}]i$ influx. As discussed in section III.A, such a mechanism could be responsible for triggering an AMPA-induced $[Ca^{2+}]i$ destabilization. A relative deficit of trophic factors in adult caudate nucleus or a lack of appropriate transduction system via trophic factor receptors in dopaminergic neurons would further facilitate apoptosis under these circumstances. Indeed, it has been demonstrated that several growth factors are able to restore mitochondrial function and prevent loss of $[Ca^{2+}]i$ homeostasis and cell death in glucose-deprived hippocampal neurons (Mattson et al., 1993). In regard to cytoprotection, naturally occurring and semisynthetic gangliosides were found to protect trophic factor-deprived neurons (Ferrari et al., 1993). Thus, it is possible that trophic factors as well as gangliosides could play a common role in maintaining $[Ca^{2+}]i$ homeostasis in dopaminergic neurons. Their lack might render dopaminergic neurons vulnerable to abusive stimulation of excitatory aminoacid receptors. This model also explains the delayed appearance, as well as the progressive nature, of dopaminergic cell loss in PD, as opposed to exogenous toxicant models that induce acute cell death of a limited group of neurons.

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