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COMMENTARY

NEUROPROTECTIVE THERAPEUTIC STRATEGIES

COMPARISON OF EXPERIMENTAL AND CLINICAL RESULTS

MANFRED GERLACH,*† PETER RIEDERER*‡ and MOUSSA B. H. YOUDIM§

*Department of Clinical Neurochemistry, University Psychiatric Clinic, University of Würzburg, Germany; †Department of Clinical Neurochemistry, University Neurological Clinic, St. Josef's Hospital, Bochum, Germany; and §Faculty of Medicine, Department of Pharmacology, Technion—Israel Institute of Technology, Haifa, Israel

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In the context of neurodegenerative disorders, neuroprotective therapy refers to interventions that are designed to preserve the integrity and function of vulnerable neurons in order to prevent the manifestation of the disease, or at least to slow down or halt the progressively disabling course of the disease.

Degenerative diseases of the CNS frequently have a predilection for specific cell populations. For example, Alzheimer's disease involves many neuronal populations, but relatively early in its course it appears to affect cholinergic projections from the basal forebrain [1, 2]. By contrast, Parkinson's disease has a more dramatic predilection for the dopaminergic neurons of the substantia nigra but can also affect cell populations using other monoamines as neurotransmitters (for a review, see Refs. 3 and 4). Although many different hypotheses have been advanced, the reasons for the selective vulnerability of neuronal cell populations remain elusive as yet. The partial elucidation of the processes that underlie the selective action of neurotoxic substances, such as 6-hydroxydopamine, glutamic acid, kainic acid, quinolinic acid or MPTP‖ [5–7], has, however, revealed possible molecular mechanisms that give rise to illnesses (Table 1). Neuroprotective strategies corresponding to these concepts have been developed (Table 1), and in experimental models various substances were shown to exert a neuroprotective effect (Table 2).

Selegiline (L-deprenyl: its chemical name is variously given as isopropylphenylmethylpropargylamine, *N*,2-dimethyl-*N*-propynyl-2-phenylethylamine, *R*-(-)-*N*-methyl-*N*-1(1-phenyl-2-propyl)-2-propynylamine hydrochloride or *n*-propynyl-methamphetamine) and vitamin E (α-tocopherol) were the first substances with which the clinical demonstration of a neuroprotective effect was supposed to be achieved in prospective controlled clinical studies [32–34]. The results obtained so far could not, however, unequivocally demonstrate a neuroprotective effect in patients with Parkinson's disease [34].

In this paper we discuss the relevance of the proposed molecular mechanisms underlying the pathogenesis of neurodegenerative disorders. In addition, we give a review of preclinical results that show neuroprotective effects of representative drugs in experimental models, and the results of clinical trials with selegiline and vitamin E in the treatment of Parkinson's disease. Finally, we also discuss the problems associated with carrying out clinical neuroprotective studies, using as an example Parkinson's disease, the most thoroughly investigated neurodegenerative disease.

PROPOSED MOLECULAR MECHANISMS UNDERLYING NEURODEGENERATIVE DISORDERS

Oxidative stress

Parkinson's disease is characterized in pathological terms by a progressive loss of catecholaminergic neurons in the brainstem [3]. The degeneration of the melanin-pigmented neurons of the substantia nigra pars compacta and the resulting dopamine deficiency in the striatum are the neuropathological basis of the movement disorders characterizing Parkinson's disease [4]. The cause of the degeneration of the nigrostriatal dopaminergic neurons in Parkinson's disease is still unknown. However, there are suggestions that degeneration of these neurons is associated with and may even be due to an active toxic process involving highly reactive oxygen species such as hydroxyl ($^{\cdot}OH$) and superoxide ($^{\cdot}O_2^-$) free radicals, and nitric oxide [4, 8–10, 35, 36].

The "oxidative stress" hypothesis infers an imbalance between the formation of cellular oxidants and the antioxidative processes. Oxidative stress, due to the excessive formation of hydrogen peroxide and

‡ Corresponding author: Prof. Dr. P. Riederer, Klinische Neurochemie, Universitäts-Nervenklinik, Füchsleinstraße 15, D-97080 Würzburg, Germany. Tel. 49-931-203318; FAX 49-931-203358.

‖ Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; BOAA, β-*N*-oxalylamino-L-alanine; MAO, monoamine oxidase; MPP^+, 1-methyl-4-phenylpyridinium cation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; and SOD, superoxide dismutase.

Table 1. Possible molecular mechanisms of neuronal cell death

Molecular mechanisms	Possible causative processes	Neuroprotective strategies
Oxidative stress [5, 8–10]		
Neurotoxic effects of oxygen-derived free radicals	Metabolism of catecholamines and endogenous or exogenous neurotoxins, impaired free-radical scavenging systems, altered brain iron metabolism, postischemic reperfusion, inflammatory cytokine-induced gliosis	Antioxidative strategies with monoamine oxidase inhibitors, iron chelators, vitamins C and E Antiinflammatory drugs
Excitotoxic mechanism [6, 7]		
Excitatory amino acid receptor-mediated influx of cations gives rise to neurotoxic effects	Abnormal glutamate accumulation, exogenous excitotoxins (e.g. domoic acid, β-*N*-oxalylamino-L-alanine)	Glutamate-receptor antagonists, calcium-channel blockers
Disturbance in mitochondrial energy metabolism [5, 11–13]		
Diminished or completely ceased ATP synthesis	Mitochondrial toxins (MPP^+, paraquat, nitric oxide), glutamate-induced processes, disturbance of calcium homeostasis	Normalization of mitochondrial energy metabolism (e.g. ubiquinone supplementation), glutamate-receptor antagonists, calcium-channel blockers
Disturbance of intracellular calcium homeostasis [5, 14]		
Excessive activation of calcium-dependent enzymes (e.g. protein kinase C, phospholipases, proteases, nitric oxide synthase) that are involved in neuronal function	Cell membrane damages, impaired energy supply (e.g. inhibition of mitochondrial respiratory chain enzymes), excitotoxins	Calcium-channel blockers

oxygen-derived free radicals, can cause cell damage through chain reactions of membrane lipid peroxidation and/or alterations in membrane fluidity [8, 10]. Hydrogen peroxide is produced in human tissues by several enzymes, such as SOD (superoxide:superoxide oxidoreductase; EC 1.14.1.1), L-amino acid oxidase, glycollate oxidase, and MAO (amine:oxygen oxidoreductase (deaminating; flavin-containing); EC 1.4.3.4) [8].

In dopaminergic nerve cells, it is mainly generated by MAO via deamination of dopamine, and non-enzymatically by auto-oxidation of dopamine (Fig. 1). Hydrogen peroxide is relatively inert and not toxic to cells [8]. However, damage is done when hydrogen peroxide interacts with the reduced forms of transitional metal ions, e.g. iron(II) or copper(I), and decomposes to the highly reactive hydroxyl free radical (the Fenton reaction) (Fig. 1). However, hydroxyl radicals are also produced in the mitochondria of nerve cells during oxidative phosphorylation, as shown in equation 1 below.

$$O_2 \xrightarrow{+e^-} {}^{\cdot}O_2^- \xrightarrow[+2H]{+e^-} H_2O_2 \xrightarrow{+e^-} {}^{\cdot}OH + OH^- \xrightarrow[+2H]{+e^-} 2H_2O \quad (1)$$

Hydroxyl radicals rapidly react with and have a strong affinity for almost every molecular species found in living cells. Such reactions include breakage of single- and double-stranded DNA, and chemical alterations of the deoxyribose purine and pyrimidine bases, membrane lipids and carbohydrates [8], leading to a cascade of events with subsequent damage to the mitochondrial electron-transport system, decompartmentalization of intracellular calcium homeostasis, induction of proteolysis by proteases, increased membrane lipid peroxidation, and finally cell death (Fig. 2).

Although the proof that oxidative stress actually causes the loss of dopaminergic neurons in patients with Parkinson's disease is lacking, there is a considerable body of indirect evidence from studies in both animals and humans that supports this concept. This includes a shift of the iron(II)/iron(III) ratio in the substantia nigra from almost 2:1 in the normal brain to 1:2 in the Parkinsonian brain [37], decreased glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase; EC 1.11.1.9) and catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase; EC 1.11.1.6) activities, an increased superoxide dismutase (superoxide:superoxide oxidoreductase; EC 1.15.1.1) activity, and a decreased content of reduced glutathione (reviewed in Refs. 4 and 38–40). In addition, an increase in the basal levels of thiobarbituric acid-reactive substances (a measure of secondary products of lipid peroxidation) coupled with a decrease in the levels of polyunsaturated fatty acids (the substrates for lipid peroxidation) has been shown in the substantia nigra of patients with Parkinson's disease [41]. Measurements of more specific peroxide products, lipid hydroperoxides, have confirmed these results: a 10-fold increase in the levels of lipid hydroperoxides has been found in the substantia nigra in Parkinson's disease [42].

Table 2. Neuroprotective effects of drugs in experimental models*

Drug	Mode of action	Experimental model	References
Selegiline	Monoamine oxidase inhibition, stimulation of superoxide dismutase, induces synthesis of neurotrophic factors	6-Hydroxydopamine model in the rat MPTP model in the mouse and ape	[15] [15]
Desferrioxamine	Iron chelator	6-Hydroxydopamine model in the rat	[16]
Vitamin E	Antioxidant	6-Hydroxydopamine model in the rat MPTP model in the mouse and ape† glutamate-induced toxicity in a neuronal cell line	[16, 17] [5] [18]
GM1 ganglioside	Neuritogenic action Neurotrophic effects	6-Hydroxydopamine model in the rat MPTP model in the mouse and ape	[19] [20, 21]
U74006F	Lipid peroxidation inhibitor	Rat middle cerebral occlusion model	[22]
Dihydrolipoate	Antioxidant	Rat middle cerebral occlusion model	[23]
L-Kynurenine	Glutamate-receptor antagonist of the glycine site	Hypoxia-ischemia in the neonatal rat NMDA-induced neurotoxicity in the neonatal rat	[24]
MK-801 (dizocilpine)	Non-competitive NMDA-receptor antagonist	Methamphetamine model in the mouse and rat NMDA-induced toxicity in isolated chick embryo retina	[25, 26] [6]
Memantine	Non-competitive NMDA-receptor antagonist	Glutamate-induced neurotoxicity in fetal cortical cultures	[27, 28]
Nimodipine	L-Type calcium-channel blocker	NMDA-induced neurotoxicity in fetal neuronal cultures, MPTP model in the mice and ape‡	[29]
Veratridine	Calcium and sodium-channel blocker	Glutamate-induced neurotoxicity in fetal neuronal cell cultures	[30]
HA1077	Calcium-channel blocker	Anoxia in hippocampal neuronal cell cultures	[31]

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; and NMDA, *N*-methyl-D-aspartate.

* The list is not exhaustive, and only representative compounds are shown.

† The reported results are contradictory.

‡ Kupsch A, Gerlach M, Pupeter SC, Sauer HJ, Dirr A, Arnold G, Opitz W, Przuntek H, Oertel WH and Riederer P. Manuscript submitted for publication.

Further evidence for the occurrence of oxidative stress in Parkinson's disease comes from studies on experimental models of this disease. For example, the iron chelator desferrioxamine (desferal) and vitamin E (Table 2) protect rats against the 6-hydroxydopamine-induced reduction in striatal dopamine content and decrease of dopamine-related spontaneous locomotor activity. These findings indicate the prevention of 6-hydroxydopamine-induced degeneration of nigro-striatal dopaminergic neurons. 6-Hydroxydopamine is thought to induce nigro-striatal dopaminergic lesions via generation of hydrogen peroxide and hydroxyl radicals derived from it [43–45], presumably initiated by a transition metal such as iron. In fact, it has been shown by magnetic resonance (MR) imaging studies that iron is increased in the striatum of 6-hydroxydopamine-lesioned rats [46]. Furthermore, 6-hydroxydopamine releases iron from ferritin *in vitro* [47]. Finally, intranigral injections of iron(III) produce neurotoxic effects similar to those observed with 6-hydroxydopamine [48–50].

Although earlier data showed that MPTP-induced dopaminergic neurotoxicity does not involve oxidative stress (reviewed in Ref. 5), Chiueh *et al.* [51] recently presented convincing evidence that the neurotoxic action of MPTP involves the generation of hydroxyl free radicals from released dopamine. Additionally, Adams *et al.* [52] demonstrated that hydrogen peroxide and hydroxyl radicals are also products of the interaction of MPP^+ (probably the ultimate toxic agent) with mitochondrial NADH dehydrogenase (NAD[P]H:ubiquinone oxidoreductase; complex I; EC 1.6.99.3). The neurotoxic effects of MPTP [induction of a Parkinson-like akinesia in monkeys, which is accompanied by a striatal dopamine deficiency and a diminished cell number of neurons showing tyrosine hydroxylase (EC 1.14.16.1) immunoreactivity] can be completely prevented by prior treatment with the selective MAO-B inhibitor selegiline (Table 2), which prevents the metabolism of MPTP to the neurotoxic MPP^+. Lipid membrane-constituents such as GM1-gangliosides (Table 2) exert only a partial neuroprotective action. On the other hand, the neuroprotective actions of vitamins C and E are uncertain (see Ref. 5).

The most convincing indication, thus far, of a causal relation between an oxidative process and a neurodegenerative illness has come from genetic investigations of the SOD gene in familial ALS, a lethal progressive degenerative disease in which the

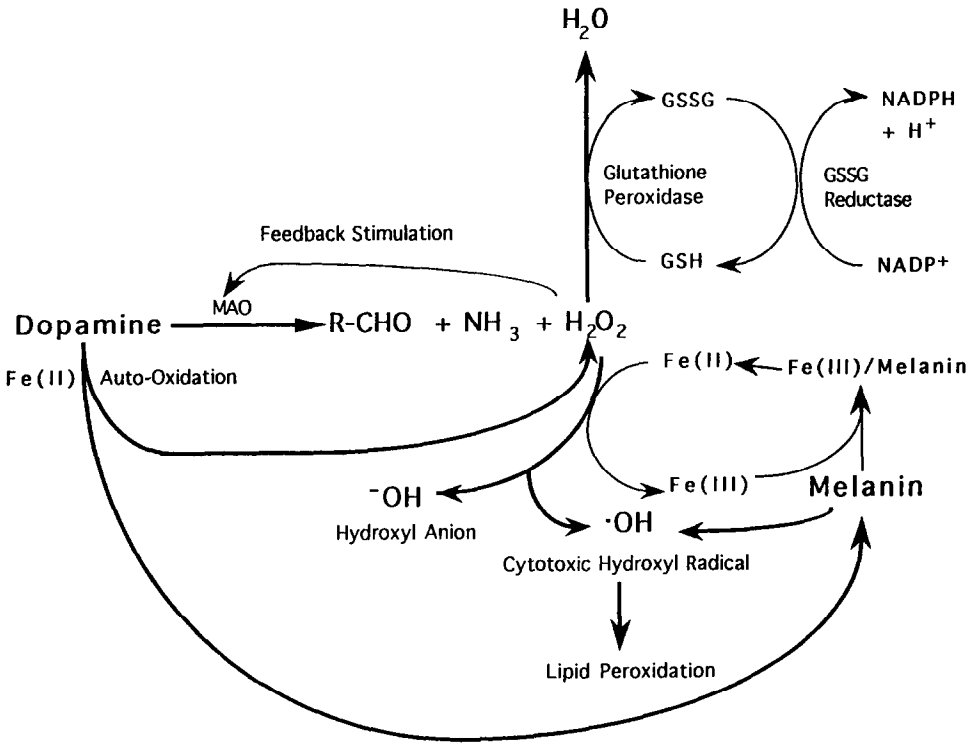

Fig. 1. Reaction pathway illustrating the ability of hydrogen peroxide and melanin to alter the redox state of iron between its two valencies with resultant formation of cytotoxic hydroxyl radical and induction of lipid peroxidation (modified according to Ref. 135).

motor neurons in the spinal cord, brainstem and motor cortex decay. Rosen and co-workers [53] have reported recently that patients with ALS showed 11 different point-mutations in the gene for the cytosolic copper/zinc SOD (SOD_1), which is located on chromosome 21. Since these changes were not demonstrable in any of the 100 controls investigated and therefore cannot be regarded as normal allelic variants, the authors concluded that these mutations to the SOD_1 gene are responsible for causing familial ALS. The Cu/Zn SOD protein is located in the cytosol and in the peroxisomes, and is a member of an SOD family that also includes the mitochondrial Mn-dependent SOD_2 and the extracellular SOD_3 [54]. These enzymes convert the toxic superoxide anion radical $\cdot O_2$ into hydrogen peroxide. SOD activity in red blood cells lysates of the family members carrying the mutation was less than half that of members without the mutation [55]. By contrast, in patients with sporadic ALS and age-and sex-matched controls, red blood cell SOD activity was normal. These studies indicate that this SOD activity is reduced in familial ALS patients but not in sporadic ALS patients. Moreover, this SOD enzyme abnormality is detectable years before the onset of clinical ALS in carriers of the familial ALS mutations.

It has been known for some time that glia, reactive microglia, and activated macrophages can also induce severe tissue oxidative stress [56–58] and be cytotoxic to neurons [59]. Macrophages are also cytotoxic to human tumor cells in culture, resulting in inhibiton of metabolic functions [60–64]. Such a phenomenon also has been stressed repeatedly in neurodegenerative disorders, especially in Parkinson's disease [65, 66]. The mechanism by which microglia and macrophages lead to metabolic inhibition and finally neuronal death is not known but must be associated with some endogenously released product. Cytokines and arachidonic acid are a few candidates. However, evidence now is accumulating to suggest that nitric oxide generation from L-arginine by such cells could also contribute to and even initiate the cytotoxic effects and neuronal cell death (reviewed in Refs. 67–69).

Biosynthesis of nitric oxide involves an oxidation of L-arginine by NADPH and molecular oxygen catalyzed by a monooxygenase called NOS (EC 1.12.23). It has been shown that its enzyme exists in at least three isoforms that were classified on the basis of their regulation by Ca^{2+} and their subcellular localization [70–72]. Since non-heme iron(II) may be involved in the electron transfer to oxygen, the shift in the ratio of iron(II)/iron(III) in the substantia nigra from about 2:1 in the normal brain to 1:2 in the Parkinsonian brain [37] may also reflect a higher synthesis of nitric oxide, possibly by reactive microglia. NOS is not confined to macrophages and microglia cells but exists in a host of cell types including neurons [73]. In the rat brain, immunocytochemical studies localized NOS to selective neuronal populations including basket and granule cells of the cerebellum; neurons within the pedunculopontine

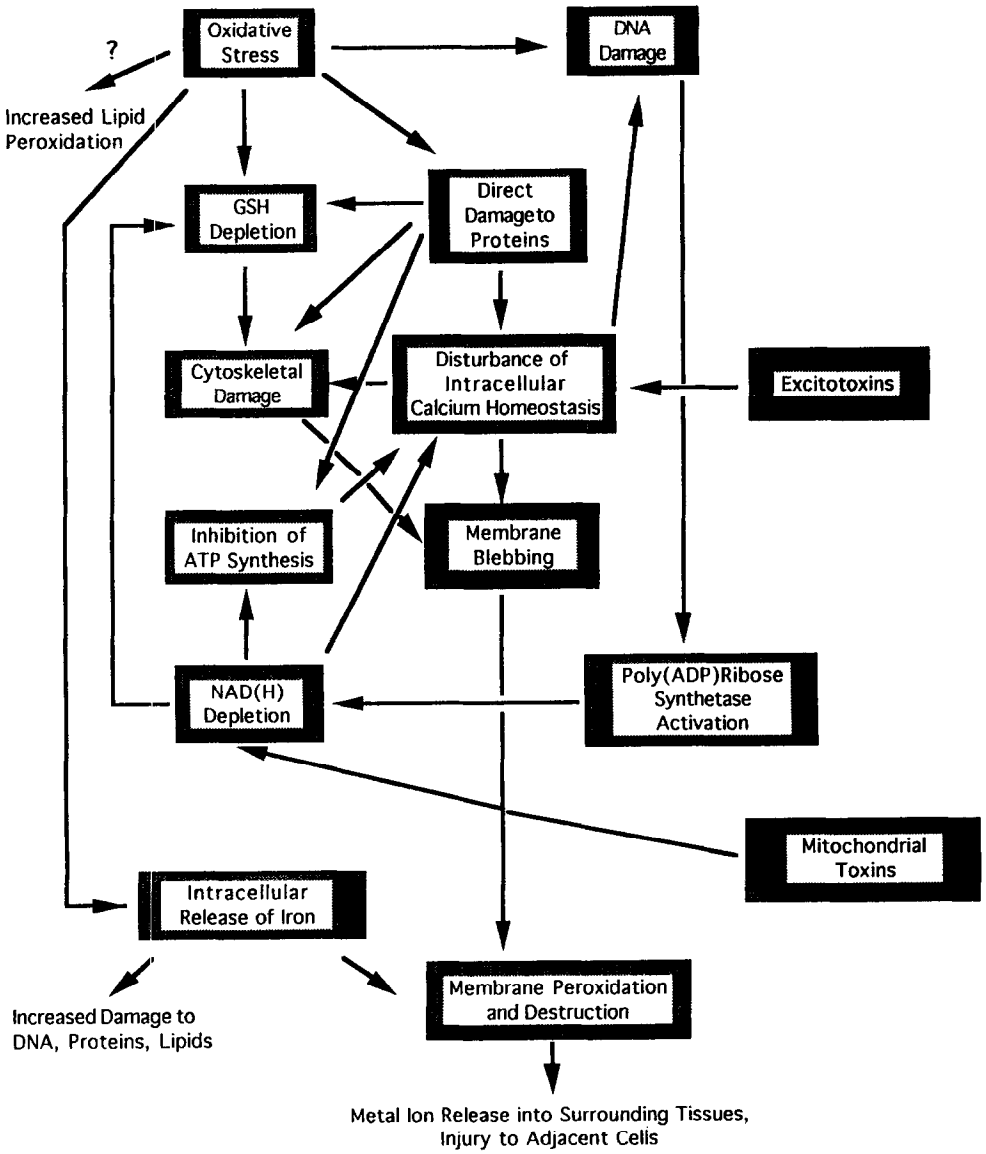

Fig. 2. Interacting mechanisms of cell injury by oxidative stress, excitotoxins, and toxins poisoning mitochondrial respiratory chain enzymes (modified according to Ref. 8).

tegmental nucleus; and medium to large aspiny neurons within the corpus striatum, cerebral cortex, and numerous other sites [73].

The mechanism by which nitric oxide kills cells and expecially neurons is not fully understood. On the one hand, toxicity may involve nitric oxide itself (Fig. 3). It is known that nitric oxide inhibits several enzymes including complex I, complex II (succinate: ubiquinone oxidoreductase; EC 1.3.99.1) and complex IV (cytochrome *c* oxidase, EC 1.9.3.1) of the mitochondrial electron transport complex [60–63, 74]; the citric acid cycle enzyme, aconitase [62]; and the rate-limiting enzyme in DNA replication, ribonucleotide reductase [75, 76]. Complex I activity has been reported to be decreased also in the substantia nigra of the Parkinson's disease brain and appears to be anatomically specific to the substantia nigra and disease-specific to Parkinson's disease (Reviewed by Ref. 11). All these enzymes have a catalytically active non-heme iron–sulfur complex. The ability of activated macrophages to inhibit non-heme iron-dependent mitochondrial enzymes as well to synthesize nitric oxide led Lancaster and Hibbs [64] to suggest a direct interaction between iron and nitric oxide. Indeed, they were able to provide evidence for iron–nitrosyl complexes in cytotoxic activated-macrophage effector cells. The relatively large amount of iron decompartmentation led Wharton *et al.* [63] to speculate that some of the iron may arise from its storage site in the protein ferritin, since ferritin can store up to 4500 atoms of iron per molecule as iron(III). Indeed, Reif and Simmons

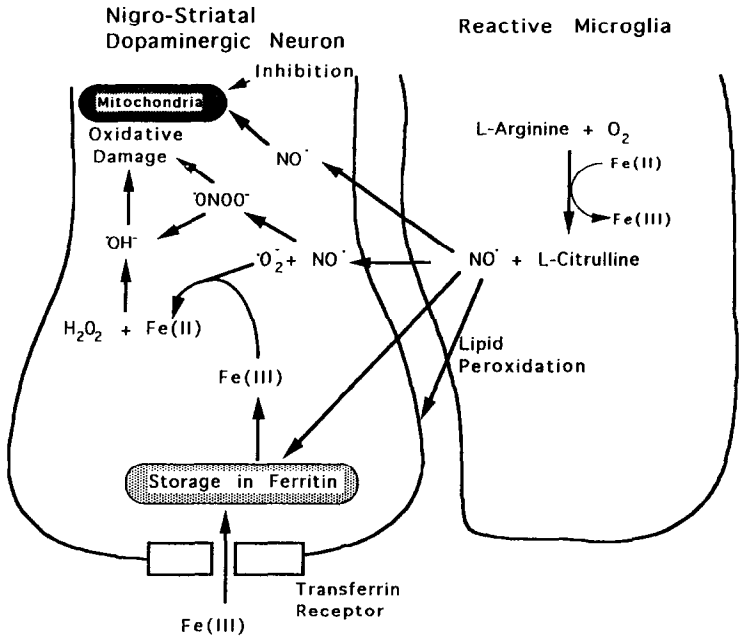

Fig. 3. Diagram illustrating the interacting biochemical events of nitric oxide (NO) and iron in the expression of neurotoxicity in Parkinson's disease (modified according to Ref. 9).

[77] and Youdim and Riederer* have shown that the addition of a nitric oxide-generating agent, sodium nitroprusside, to the iron-storage protein ferritin resulted in the release of iron from ferritin (measured spectrophotometrically as an iron(II)/ferrozine complex) and promotion of lipid peroxidation. Furthermore, as a functional antagonist to nitric oxide [78], hemoglobin [77] and desferrioxamine* inhibited these processes. The authors suggested that nitric oxide generation *in vivo* could lead to the mobilization of iron ferritin, disrupting intracellular iron homeostasis, and increasing the level of reactive oxygen species with consequential lipid peroxidation (Fig. 3).

On the other hand, nitric oxide as a fee radical *per se* is a highly reactive molecule that can react with molecular oxygen and the superoxide anion (Fig. 3). The last reaction results in the formation of the peroxynitrite anion ($^{\cdot}ONOO^{-}$), which is described as an extremely reactive molecule with potent oxidant properties [79, 80]. The peroxynitrite anion decomposes to the hydroxyl free radical and to nitrogen dioxide (Fig. 3), which are potent activators of lipid peroxidation [79, 80]. However, recent observations in human leukocytes [81] indicate that nitric oxide can be regarded as a scavenger of superoxide anion and suggest that nitric oxide may provide as a chemical barrier to cytotoxic free radicals. Experiments with transgenic mice overexpressing human extracellular SOD led to the conclusion that SOD increases CNS oxygen toxicity by inhibiting superoxide anion-mediated inactivation of nitric oxide [82].

Excitotoxic mechanism

The concept of excitotoxic cell death postulated by Olney [83] implies two paradoxical modes of action of amino acid neurotransmitters (e.g. glutamic acid, aspartic acid) or of excitatory amino acid receptor agonists (e.g. kainic acid, quinolinic acid), namely that they exert an excitatory action and at the same time a neurotoxic effect. Excitatory amino acids bind to specific receptor binding sites, and operate to stimulate neurons by opening receptor-coupled ion channels [84]. This action is essential for the normal functional activity of the CNS. In the case of a neurotoxic effect, there is a massive increase of the intracellular calcium concentration as a result of the excessive stimulation of glutamatergic neurons.

The concept of excitotoxic cell death (for a review, see Refs. 6, 7 and 36), which has in recent years received experimental verification points to possible mechanisms of how not only endogenous transmitters (glutamic acid, for example), but also exogenous neurotoxins (for example BOAA) can damage neurons. It is recognized nowadays [6, 7] that the receptors that are particularly involved in the toxic effects of excitatory amino acids are both the subtypes of the glutamate receptor, the NMDA receptor and AMPA receptor. It is possible to imitate the neuropathological correlates of Huntington's disease (loss of GABAergic and cholinergic markers in the striatum) in animal experiments by the injection of quinolinic acid, an endogenously occurring

* Youdim MBH and Riederer P, Neurotoxicity of nitric oxide and decompartmentation of ferriton-iron. *Proceedings of the 14th ISM*, Montpellier, 1993.

Table 3. Neurodegeneration syndromes associated with excitatory amino acids (according to [6])

Disease	Excitotoxin and excitotoxic mechanism, respectively
Food excitotoxins	
Neurolathyrism	β-*N*-Oxalylamino-L-alanine (BOAA)
Guam disease [amyotrophic lateral sclerosis (ALS)/ Parkinsonism/dementia complex]	β-*N*-Methyl-amino-L-alanine (BMAA)
Metabolic disorders	
Sulfide oxidase deficiency	Cysteine-*S*-sulfate
Olivopontocerebellar atrophy (OPCA)	Glutamate, because of a postulated defect in CNS catabolism of glutamate
ALS	Glutamate, because of a postulated unknown metabolic defect
Energy deficiency disorders	
Epilepsy	Partial exhaustion of energy resources in the microenvironment of the NMDA receptor is a responsible factor in seizure-related brain damage
Hypoxia/ischemia	Excessive release of glutamate
Chronic neurodegenerative diseases	
Alzheimers' disease	Glutamate/aspartate?
Huntington's disease	Glutamate? Quinolinate?
Parkinson's disease	Glutamate?

NMDA agonist [85, 86]: The conclusion reached was that an increased rate of synthesis of glutamic acid or an increased sensitivity of cortical glutamatergic neurons was causally responsible for this neurodegenerative disease [87, 88], which is inherited via the autosomal dominant mode (chromosome 4) with complete penetrance.

Although patients who had died with Huntington's chorea had an up to 5-fold increase in brain activity of the enzyme 3-hydroxyanthranilic acid oxygenase, the enzyme responsible for synthesizing quinolinic acid, compared with controls [89], increased concentrations of quinolinic acid could not be demonstrated in urine [90] or cerebrospinal fluid [91, 92] nor in the brain itself [92, 93]. *Post mortem* investigations of the NMDA receptor [94], which showed a decrease in glutamate binding in the putamen (−97%) of patients with Huntington's disease, but not in the frontal cortex, might, however, indicate a possible role for excitatory amino acids in the pathogenesis of this disease. Table 3 gives further examples of neurodegenerative diseases in which an excitotoxic effect is discussed.

The excessive stimulation of the NMDA receptor by glutamic acid or NMDA agonists leads to a massive influx of calcium ions into the cell; the activation of calcium-dependent enzymes sets in action mechanisms that lead to the decline of the cell (for a review, see Refs. 7, 35, 36). For example, the activation of calpain I and II leads to alterations in the cytoskeleton; the activation of protein-kinase C and nitric oxide synthase results in the formation of toxic free radicals (Fig. 4); the activation of phospholipase A_2 leads to the breakdown of phospholipid membranes. The fatty acids liberated by this process, such as arachidonic acid, then get into the extracellular space and are broken down further into free radicals. In this way, a vicious circle of cell damage is maintained or even reinforced (Fig. 4). In this connection it is interesting to note the recently described finding according to which the glutamate of astrocytes cultured from embryonic rat or mouse striatum is capable of releasing arachidonic acid [95]. Thus, glutamate-evoked release of arachidonic acid seems to be regulated in opposite fashion by protein kinases A and C, because stimulation of adenylate cyclase by the β-adrenergic agonist isoproterenol, vasocative intestinal peptide or pretreatment of the striatal astrocytes with cholera toxin decreased the glutamate-evoked release of arachidonic acid. By contrast, ATP, which markedly stimulated inositol phosphate production, strongly potentiated the glutamate-evoked release of arachidonic acid [95].

The ligand-coupled activation of the NMDA receptor, however, does not lead to an influx of only calcium ions, but sodium ions also enter the cell, which produces a strong depolarization of the neuron [96]. This results in an additional massive calcium influx through the opening of the "voltage-dependent" type-L ion-channels.

In cultured cells and in animal experiments it is possible to prevent the toxic action of excitatory amino acids by the administration of non-competitive NMDA-receptor antagonists and by calcium-channel blockers, which inhibit the entry of calcium ions (Table 2).

Disturbance in mitochondrial energy metabolism

The mitochondria supply cells, including neurons, with energy (for a review see Ref. 13). High-energy carrying compounds such as $NADH_2$ and $FADH_2$ are formed during the Krebs cycle within the mito-

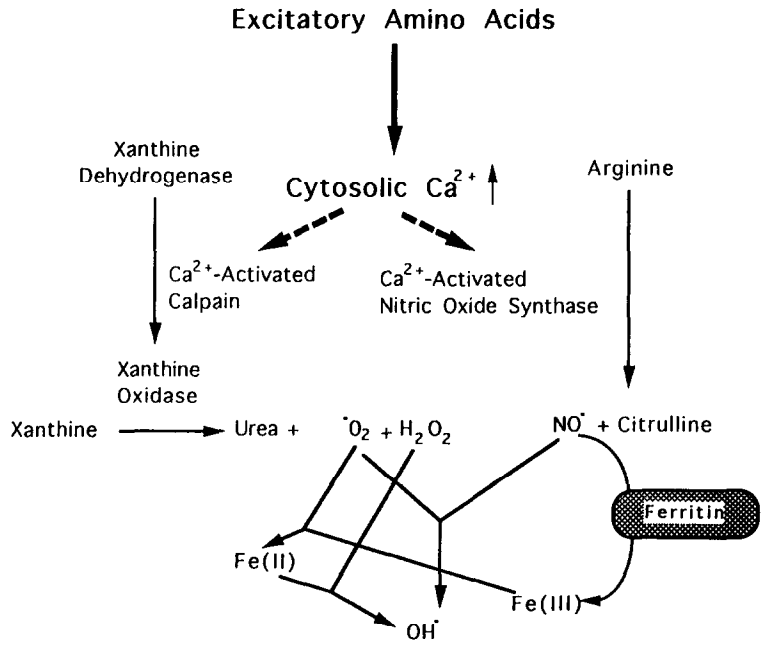

Fig. 4. Cascades of molecular events leading to neuronal cell death (modified according to Ref. 35).

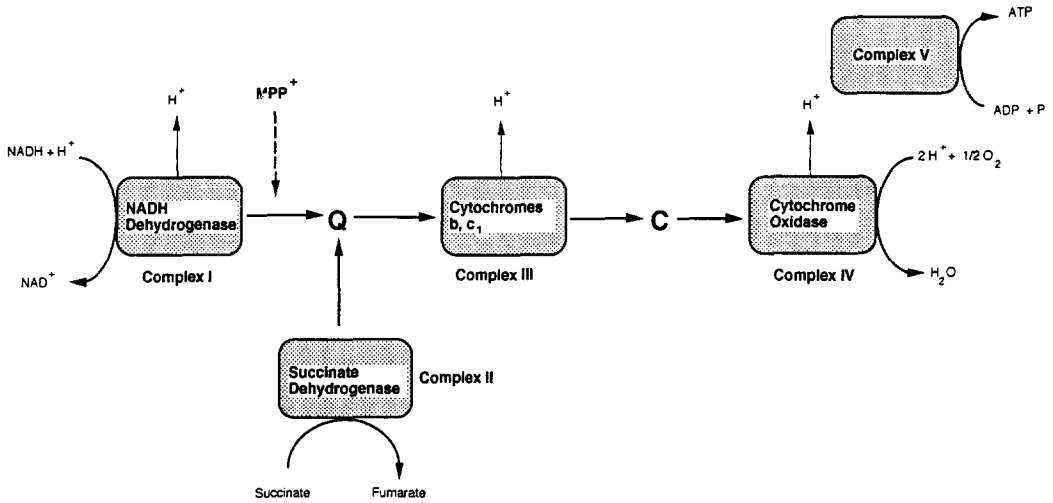

Fig. 5. Schematic representation of the effect of MPP^+ on the respiratory chain (modified according to Ref. 5). C, cytochrome *c*, and Q, ubiquinone.

chondrial matrix. $NADH_2$ and $FADH_2$ donate electrons that are required for the generation of an electrochemical gradient across the inner membrane of the mitochondrion and thus for the synthesis of ATP. The enzymes responsible for electron transport are located in the inner mitochondrial membrane. Two very important members of this highly organized and hierarchical system of enzymes are NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) (Fig. 5). The electrons entering the system are transported via flavin mononucleotides (FMN) and the multiple iron–sulfur centres of complex I or II and passed to ubiquinone (coenzyme Q, CoQ). The electrons are then transported to the ubiquinol:cytochrome *c* oxidoreductase (complex III), which contains cytochrome *b*, Rieske iron-sulfur protein, and cytochrome c_1. From complex III electrons are transferred via cytochrome *c* to cytochrome *c* oxidase (complex IV) containing cytochromes *a* and a_3 equipped with copper atoms, and finally passed to oxygen (Fig. 5). As electrons pass through complexes I/II, III and IV, protons

cross the inner mitochondrial membrane, creating an electrochemical gradient. This energy is used by ATP synthase (complex V) for the synthesis of ATP. The adenine nucleotide translocator then exchanges ATP for cytosolic ADP.

Poisoning of oxidative phosphorylation disturbs the function of the mitochondrial respiratory chain, and results in a depletion of the ATP stores in the mitochondria and in the cytoplasm. MPP^+, probably the ultimate neurotoxin responsible for degeneration of dopaminergic neurons, is a specific complex I inhibitor, blocking one of the two major pathways for electrons entering the mitochondrial respiratory chain (Fig. 5). Aminooxyacetic acid (AOAA), an excitotoxin, interrupts the supply of electrons to mitochondrial NADH, and this leads indirectly to a relative insufficiency of complex I since reducing equivalents required for oxygenation do not enter the respiratory chain [97]. The intrastriatal application of this non-selective inhibitor of transaminases gives rise to axon-sparing lesions in the rat striatum, similar to quinolinic acid, which can be blocked by NMDA agonists and by prior decortication [97].

The selective inhibition of complex I activity by high concentrations of MPP^+ (1 mM) demonstrated in both heart and liver mitochondria might be considered as the sole cause for the neurodegenerative effect seen after systemic treatment with MPTP, but the latest investigations have also shown that this inhibition of complex I additionally leads to the mitochondrial production of superoxide free radicals [98, 99], hydrogen peroxide and hydroxyl radicals [52]. Inhibition of complex I by paraquat likewise leads to the formation of superoxide radicals [100]. Interestingly, it was shown recently that 6-hydroxydopamine, a neurotoxin that is thought to induce nigro-striatal dopaminergic lesions via generation of hydrogen peroxide and hydroxyl radicals derived from it [43–45], is even more toxic to complex I than MPP^+*. What is also particularly interesting in this connection is the finding that in patients with Parkinson's disease a diminished activity of complex I could be demonstrated specifically in the substantia nigra (for a review, see Ref 11). Experiments on rats showed [101] that chronic treatment with levodopa (L-3,4-dihydroxyphenylalanine, the metabolic dopamine precursor capable of crossing the blood–brain barrier, which is used in the symptomatic treatment of Parkinson's disease) leads to reversible inhibition of complex I activity, but not of the activities of complexes II and IV. Incubation of whole brain mitochondria *in vitro* showed that both levodopa and dopamine inhibit complex I activity in a dose- and time-dependent manner, whereas other metabolites of levodopa were minimally effective. Reduced glutathione, ascorbate, SOD and catalase prevented the effect of dopamine. In agreement with a critical role of MAO in the effect *in vitro*, other non-catecholamine substrates of this enzyme, such as serotonin and β-phenylethylamine, were potent inhibitors of complex I [101]. However, auto-oxidation may also be involved in this process, because the effects of levodopa, 6-hydroxydopa and 6-hydroxydopamine on complex I activity were only partially suppressed by MAO inhibitors [101]. These observations demonstrate that the chronic administration of levodopa can cause alterations in mitochondrial respiratory chain activity in rats, and that these changes are most likely related to oxidative stress provoked by the increase in dopamine turnover.

Disturbance of intracellular calcium homeostasis

The calcium hypothesis of necrotic cell death postulates that whenever pathologically increased intracellular calcium concentrations occur, there will be an uncontrolled stimulation of calcium-dependent enzyme reactions, and that these will lead to altered cell function and the destruction of cellular structures [14]. It was originally assumed that the excessive calcium influx was caused by "voltage dependent" ion channels, but a calcium influx coupled to the NMDA receptor and/or free radical-induced membrane damage could equally lead to a breakdown of calcium homeostasis (Fig. 2). Thus it can be argued that the breakdown of intraneuronal calcium homeostasis represents an ultimate pathobiochemical mechanism that may also occur as the result of these previously described mechanism (induced by free radicals or excitotoxins through interference with the mitochondrial energy supply; see Figs. 3 and 4). In the light of this hypothesis, it appears distinctly possible that the effects of various neurotoxins exerting their action via a variety of different pathological mechanisms could all be prevented by calcium-channel blockers. In fact, in animal experiments using the MPTP model in the mouse and in the monkey, a neuroprotective effect was indeed demonstrated with the calcium-channel blocker nimodipine (Table 2). Similarly, in primary neuronal hippocampal cell cultures, glutamate-induced toxicity was prevented by the novel calcium-channel blocker KB-2796 [102]. Furthermore, it could be shown that (*S*)-emopamil (a novel calcium-channel blocker of the phenylalkylamine type and a serotonin antagonist) significantly improved post-ischemic hypoperfusion and metabolic impairment and protected against ischemia-dependent damage to nerve cells [103]. To provide further support for this hypothesis, however, it will be necessary to carry out investigations with other calcium-channel blockers and with other neurotoxins such as 6-hydroxydopamine, methamphetamine, kainic acid or quinolinic acid.

There are indeed findings in a whole range of other neurodegenerative diseases that give a reason to conclude that calcium metabolism has been altered (for a survey see Refs. 104 and 105); the overall calcium concentration, however, appears to be unaltered. Thus, all the brain regions of patients who had died with Parkinson's disease were found to have unchanged calcium concentrations [106]. On the other hand, in the brains of deceased Alzheimer patients a significant loss of the calcium-binding protein calbindin D28k was reported [107], although this does not appear to be specific for this disease. Similar deficiencies were found in the substantia nigra of patients with Parkinson's disease [108]. Other intracellular calcium-binding proteins such as calmodulin, calpain and S100β, are equally affected

* Galenke Y and Youdim MBH. Manuscript submitted for publication.

in patients with Alzheimer's disease [109, 110]. A diminished density of dihydropyridine-binding sites was found in the temporal cortex of patients with Alzheimer's disease [111], in the striatum of patients with Huntington's disease [105], and in the nigrostriatal system of patients with Parkinson's disease [112]. Since it is specifically the neurons in these brain regions, respectively associated with these diseases, that degenerate, one may assume that the dihydropyridine-binding sites are pre-synaptically located on these degenerating neurons.

REASONS FOR CARRYING OUT CLINICAL STUDIES WITH SELEGILINE AND α-TOCOCPHEROL FOR THE DEMONSTRATION OF A NEUROPROTECTIVE EFFECT IN PARKINSON'S DISEASE

Various indirect evidence, as has been extensively described above, is available for the idea that oxidative stress is the cause of Parkinson's disease or at least it plays a role in the pathogenesis of the disease as a secondary factor. It is known that various enzymes (e.g. SOD, catalase), free radical scavengers (e.g. reduced glutathione, vitamins C and E), and inhibition of MAO (e.g. by selegiline) can prevent the damage caused by free radicals *in vitro* and *in vivo* (Table 2; for reviews, see Refs. 5, 8 and 10). The concept of oxidative stress as the molecular pathological mechanism of the dopaminergic neurodegeneration in Parkinson's disease is matched by the idea that antioxidant strategies should be able to prevent Parkinson's disease or at least to retard the course of the disease.

Selegiline and α-tocopherol were the first substances with which prospective controlled clinical trials were carried out with the aim of demonstrating a neuroprotective effect. The starting point for these trials was the 9-year retrospective investigation by Birkmayer and his colleagues [113] on 841 patients with Parkinson's disease, who had been treated either with levodopa alone or in combination with selegiline. A significantly longer life expectancy could be shown for the group of patients who had received selegiline and levodopa compared with those who had received levodopa alone. This result, as well as preclinical indications of a neuroprotective effect of selegiline and α-tocopherol (Table 2) together with theoretical considerations (oxidative stress as cause of nerve cell death), invited the assumption of a neuroprotective potential for these two drugs. Selegiline is a selective and irreversible inhibitor of type B MAO when administered at a dosage of 10 mg/day [114]. α-Tocopherol, a biologically active component of vitamin E, attenuates the effects of lipid peroxidation by trapping free radicals [8].

PROBLEMS DURING THE IMPLEMENTATION OF CLINICAL STUDIES ON NEUROPROTECTION WITH PARKINSON'S DISEASE AS AN EXAMPLE

The neuroprotective effectiveness of selegiline and α-tocopherol was investigated in two recently completed placebo-controlled double-blind studies on patients with hitherto untreated Parkinson's disease. The time at which it became necessary to begin symptomatic treatment with lovodopa was taken as the end-point. Tetrud and Langston [32] investigated 32 patients with Parkinson's disease over a 3-year period and found that with selegiline there was a significant deferment of the time at which symptomatic levodopa treatment became necessary. In the multicenter DATATOP study (deprenyl and tocopherol antioxidative therapy of Parkinsonism) that was initiated in 1987, the effect of α-tocopherol and selegiline was investigated in 800 patients with Parkinson's disease [33, 34]. An interim analysis of the trial after 12 ± 5 months indicated that selegiline reduced the risk of disability requiring levodopa therapy by approximately 50% [33]. However, it was unclear whether the effects of selegiline would be sustained or whether selegiline resulted in only short-term amelioration of clinical features (symptomatic effect), a slowing of underlying nigral degeneration (protective effect), or both of these mechanisms. An extended analysis after a mean (± SD) follow-up of 14 ± 6 months showed no beneficial effects of α-tocopherol, nor any interaction between α-tocopherol and selegiline. Further, the beneficial effects of selegiline, which occurred largely during the first 12 months of treatment, remained strong, and significantly delayed the onset of disability requiring levodopa therapy. The difference in the estimated median time to the end-point was about 9 months.

The improvement in the rating scores for Parkinson's disease disease after the initiation of selegiline and the worsening of motor performance during the 2 months after withdrawal suggest that the observed benefit of selegiline in delaying disability is partly related to a symptomatic amelioration of Parkinson's disease. On the other hand, the superior survival with respect to the primary end-point even among selegiline-treated subjects who initially had no improvement in total rating scores, and the overall persisting benefit (as compared with baseline status) among the selegiline-treated subjects who did not reach the end-point and who did not require selegiline during the 2 months after withdrawal, do suggest a protective influence.

Hence, a neuroprotective effect of selegiline is neither confirmed nor refuted in these clinical trials. To establish whether an amphetamine-like effect is contributing to the symptomatic action of selegiline (selegiline is metabolized, alongside other metabolic routes, to amphetamine and methamphetamine [115]), an 8-week double-blind study was carried out by "The Parkinson Study Group" [116], using the MAO-B inhibitor lazabemide (Ro 19-6327), because the chemical structure of this substance did not suggest that it would be metabolized to products with an amphetamine-like action. Four weeks after the start of treatment with lazabemide, the patients with Parkinson's disease showed a symptomatic effect similar to that with selegiline. This signified that the symptomatic effect demonstrated with selegiline was not called forth by the amphetamine-like component in its activity.

The dopamine therapy delaying effect was also demonstrated with the dopamine agonist lisuride in retrospectively organized studies (for a review see Ref. 117). The results led to the conclusion, analogous to that of the DATATOP study, that this effect was mediated via the symptomatic component

of lisuride's activity. However, it is equally plausible to conclude that here too a decreased formation of free radicals may lead to slowing down the progression of the disease, because dopamine agonists can also lead to a diminished dopamine turnover and in this way contribute to a reduced formation of oxygen radicals.

Results similar to those of the DATATOP study were also reported recently by Przuntek. This was also a placebo-controlled and multicenter longitudinal study designed to measure the effect of selegiline on the time course of changes in the required dosage of levodopa and of the levodopa-determined fluctuations in mobility (SELEDO study: selegiline and levodopa long-period trial). Interim analysis of the findings after 4 years showed significantly reduced values for both these parameters in the group treated with selegiline and levodopa compared with the group treated with levodopa alone.*

The results of the studies cited here emphasize how difficult it is to demonstrate in clinical studies on patients that a neuroprotectively active drug is actually exerting a neuroprotective effect. What makes it even more difficult is that the clinical symptoms of Parkinson's disease only begin to show up when more than two-thirds of the available dopaminergic neurons have degenerated and there is a dopamine deficit of 70–80% [118, 119]. One can thus expect clinical studies of potentially neuroprotective substances at best to halt the progression of the disease. Effective neuroprotection could only be obtained by attempting treatment at very early stages of the disease before it is clinically manifest. However, this is not possible at present, because of the inability to make an early diagnosis.

Since the effect of a potentially neuroprotective substance has, up to now, been judged only on the basis of the motor symptoms, a symptomatic effect of the substance has to be excluded in order to demonstrate a neuroprotective effect when evaluating motor performance parameters. In the sense in which the definition of a neuroprotective effect was defined in the introduction, that is to protect neurons against neurotoxic effects and to safeguard the integrity of vulnerable neurons, it is really necessary to demonstrate such effects at the cellular level. However, this is not possible with the equipment currently available. The resolving power of positron emission tomography (PET), with which one can visualize the functional activity of dopaminergic neurons in actual patients after administration of ^{18}F-levodopa, is at present not sufficiently great to display the substantia nigra, or to demonstrate a disease-related progressive diminution of ^{18}F-levodopa and ^{18}F-dopamine enrichment in the striatum of patients with Parkinson's disease [120].

SUMMARY AND FUTURE OUTLOOK

Although it has proved possible in various experimental models of neurodegenerative diseases to show that certain drugs exert a neuroprotective action at the cellular, biochemical and functional levels (Table 2), it has thus far not proved possible to demonstrate an unequivocal neuroprotective effect for any drug in clinical studies. On the one hand this is because symptomatic parameters have to be used as criteria of proof while in the evaluation of the results it is impossible to separate out the symptomatic effects of the substances being investigated from the possible conclusion of a neuroprotective effect on the basis of symptomatic changes. In principle, even a slight neuroprotective effect would need to be established at the cellular or biochemical level. However, the resolving power of the currently available imaging techniques such as PET or SPECT (single photon emission tomography) is not sufficiently great to display the relevant cellular structures or to demonstrate a progression of the disease. On the other hand, because we lack the ability to make diagnoses before the appearance of symptoms, clinical studies are at present being carried out on patients in whom the disease is already clinically apparent.

In spite of a great deal of effort to develop objective biochemical and instrumental methods for the early diagnosis of neurodegenerative disease [121–124], with the exception of the pre-symptomatic gene-diagnosis of Huntington's disease [125] there is at the moment no procedure by which these diseases can be diagnosed with certainty before their symptoms manifest themselves. In Parkinson's disease one starts off with the knowledge that on the first appearance of symptoms already at least 60% of the neurons are damaged [118, 119]. What this means is that with a neuroprotective drug of high potency the best that one can achieve for a patient is to halt the progression of the disease. Finally, one should not forget that in spite of the known molecular mechanisms that lead to cell death (Tables 1 and 3; Figs. 1–3), the actual causes of many diseases are not known. If a drug shows a neuroprotective effect in a particular model of a neurodegenerative disease, the only immediate conclusion one can draw is that the neuron-damaging effect of the toxin, corresponding to more or less well-known molecular mechanisms has been prevented. One cannot argue the reverse, that a substance which has not shown any benefit in a particular model would also fail to be neuroprotective with a different model. The ultimate proof of the effectiveness of a neuroprotective drug in a neurodegenerative disease must, in the end, be provided by a clinical trial.

Therefore, in the planning and performing of future clinical investigations, the following points should be taken into consideration:

(1) The substances to be tested should be those which, on the basis of their pharmacological profile, would be expected to have few or no symptomatic effects. These are, above all iron chelators able to cross the blood–brain barrier such as antioxidative-acting lazaroids [22], calcium-channel blockers of the dihydropyridine type such as nimodipine or nitrendipine, which are able to act on NMDA-coupled and voltage-dependent calcium channels [126], non-competitive NMDA antagonists such as memantine [127], irreversible NOS inhibitors such as *N*-nitro-L-arginine, which when given systemically can inhibit

* Przuntek H, "Seledo" longtime trial: 4 years interim analysis. Symposium "Selegeline in Perspective," Würzburg, Germany, 22 November 1993.

NOS in the brain (see Ref. 128), substances with antiphlogistic activity such as corticoids, indomethacin and aspirin, or immunosuppressive drugs such as cyclosporin A and FK 506. The reasons for testing the last-named substances for their clinical neuroprotective effectiveness are histopathological findings that point to auto-immune factors in the etiology of Alzheimer's disease and Parkinson's disease [65, 66, 129]. Reactive microglia and activated macrophages can likewise elicit oxidative stress via messenger substances such as cytokines or nitric oxide, and so lead to the destruction of neurons (for a review, see Ref. 9). Cyclosporin A and FK 506, which have been used successfully to protect against immunological reactions in transplantation surgery [130], inhibit the production of cytokines, such as interleukin-2, and in this way suppress the immune response.

(2) Clinical trails should be carried out on substances that have shown some neuroprotective activity at the cellular, biochemical and functional levels (e.g. calcium-channel blockers) in different models.

(3) Neuroprotective strategies should be tested in model experiments in Huntington's disease, a chronic neurodegenerative disease that can actually be diagnosed before the onset of symptoms, and which in comparison to Parkinson's disease or Alzheimer's disease has a more rapid progressive course over a shorter time-span.

(4) Particular subtypes of neurodegenerative diseases (for example, the rigidity-akinesia type of Parkinson's disease; the bulbar form of ALS), are known to have a more marked clinical progression, and in these a more pronounced effect may be anticipated in a shorter period of observation; therefore, in future studies patients shoud be selected accordingly.

(5) Modern methods of statistical analysis of the data should be used to evaluate the results of clinical studies. In a recent longitudinal Gompertzian analysis, the supposed neuroprotective effect of cigarette smoking on Parkinson's disease was not upheld [131]. However, it will require future clinical studies to determine whether this statistical approach was suitable for distinguishing between symptomatic and neuroprotective effects [132].

(6) Because neuroprotective therapy can only protect intact neurons against damage, in future studies greater emphasis should be placed on testing neuroregenerative strategies (for reviews, see Refs. 133 and 134). Admittedly, the practical application of neurotrophic factors such as NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), neurotrophins (NT3, NT4/5) or GDNF (glial-derived neurotrophic factor) is limited by their low blood–brain barrier permeability and by their rapid proteolytic degradation. For these reasons, pharmacological attempts to stimulate endogenous synthesis of such factors are to be preferred.

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