Prospects for new restorative and neuroprotective treatments in Parkinson's disease

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The degeneration of forebrain dopamine systems in Parkinson's disease has been an effective target for pharmaceutical research over the past four decades. However, although dopamine replacement may alleviate the symptoms of the disease, it does not halt the underlying neuronal degeneration. The past decade has seen major advances in identifying discrete genetic and molecular causes of parkinsonism and mapping the events involved in nigral cell death. This new understanding of the pathogenesis of the disease now offers novel prospects for therapy based on targeted neuroprotection of vulnerable neurons and effective strategies for their replacement.

Parkinson's disease (PD) is one of the major neurodegenerative disorders of middle and old age, and was originally described by James Parkinson in 1817. It is characterized by a trio of cardinal symptoms—muscle rigidity, tremor and bradykinesia—but can also involve postural deficits and impaired gait, as well as dementia in a significant minority of patients.

The main pathological feature of the disease was first described by Lewy in 1912 as a characteristic hyaline inclusion in the cytoplasm of neurons. These so-called Lewy bodies were subsequently demonstrated by Tretiakoff to occur most prominently in the substantia nigra. Lewy bodies are spherical inclusions 5–25 μm in diameter, seen as a dense eosinophilic core with a pale surrounding halo in the cytoplasm of affected neurons (Fig. 1)[1,2]. Although they are also found in other neurodegenerative conditions, the presence of Lewy bodies in the substantia nigra, in conjunction with nigral cell loss, is generally considered to be the defining neuropathological feature in idiopathic PD, to distinguish it from other PD-like and PD-plus motor disorders involving primary degeneration in other basal-ganglia nuclei[3,4].

Although idiopathic PD is usually sporadic, it has long been recognized that there is a genetic component to the disease. Case-control studies have typically indicated a 2–14-fold increase in incidence in close relatives of PD patients[5] and although concordance rates between identical twins are low for overt expression of the disease[6], they are much higher when subclinical decline in striatal dopaminergic dysfunction is measured by positron emission tomography (PET) imaging (53% in monozygotic twins of PD patients, compared with 13% in dizygotic cases)[7]. Nevertheless, in sporadic PD, environmental factors have been emphasized[8]. Epidemiological studies in the 1970s and 1980s revealed a geographical variation in incidence that was tentatively associated with farming practice[3,8]. More recently, the discovery of a toxin, the heroin analogue 1-methyl-4-phenylpyridinium (MPTP), causing a PD syndrome that is indistinguishable from the idiopathic disease has not only added new weight to a toxic theory of causation, but also provided an accurate primate model of the disease[8]. The discovery of gene linkage in rare families with strong patterns of inheritance of PD-like disease, and the identification of the individual genes involved (Box 1), has provided new insight into the involvement of specific genes and molecules, in particular α-synuclein, which were not previously thought to be associated with the pathogenesis of the disease.

Molecular pathology

A central role for α-synuclein in the pathogenesis of PD is suspected not only because of the identification of several different mutations in familial PD, but also because α-synuclein is a prominent constituent of Lewy bodies in idiopathic PD (Fig. 1)[2,9,10]. α-Synuclein is normally a soluble unfolded protein, but it can aggregate into insoluble amyloid fibrils which then may form Lewy bodies, followed by subsequent ubiquitination and accumulation of neurofilaments[2,10]. At high concentrations, wild-type α-synuclein will self-aggregate in solution to form Lewy-body-like fibrils and discrete spherical assemblies, and this process is accelerated in the mutant forms of α-synuclein (Box 1)[2,10,11]. A challenge now is to identify the cause of aggregation of α-synuclein in the sporadic forms of PD, and the stimuli that determine the loci of precipitation that distinguish idiopathic PD from other 'synucleinopathies' such as Lewy-body dementia (Box 2). In addition, the identification of

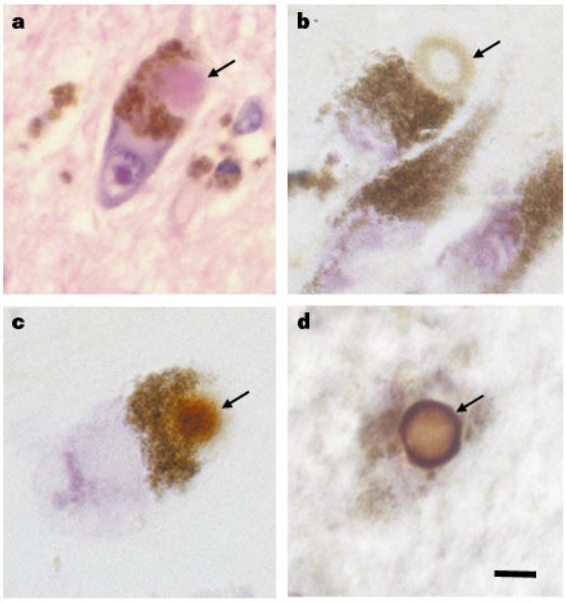


Figure 1 Lewy bodies in pigmented neurons of the substantia nigra from a PD patient, seen as large spherical inclusions in the cell cytoplasm (arrows). **a**, Haematoxylin and eosin stain. **b**, Ubiquitin immunohistochemistry which stains predominantly the halo of the Lewy body. **c**, α-Synuclein immunohistochemistry, in which the core of the Lewy body is most densely stained. **d**, Double staining with ubiquitin (blue) and α-synuclein (brown) showing additive staining of the two markers in the halo and core, respectively, of the Lewy body. The granular brown deposits in the cytoplasm of cells in each micrograph indicate the melanin pigmentation of nigral neurons, not all of which contain Lewy bodies (see **b**). Scale bar, 10 μm. (Micrographs provided by M. G. Spillantini, University of Cambridge, UK.)

Box 1 Gene mutations identified in familial PD

Gene	Chromosome	Inheritance	Phenotype	Pathology	Refs
α-synuclein	4	Autosomal dominant	L-DOPA responsive, early onset PD	Lewy bodies	93
parkin	6	Autosomal recessive	L-DOPA responsive, juvenile onset PD	No Lewy bodies	94
UCH-L1	4	Incomplete penetrance	Typical PD	Not reported	95
4p haplotype	4	Autosomal dominant	L-DOPA responsive, PD or postural tremor	Lewy bodies	96
PARK3	2	Autosomal dominant	Similar to sporadic PD	Lewy bodies	97

Several family pedigrees have been identified in which the inheritance of a PD-like disease is strong[5]. After identification of the linkage of markers on the long arm (q) of chromosome 4 at band 21 (4q21) to the disease in a large Italian family (the 'Conursi' pedigree), Polymeropoulos *et al.* mapped the gene locus and identified an A-to-G mutation in the 209th base in exon 4 of the α-synuclein gene (resulting in a substitution of alanine to threonine at position 53 in the protein) in this family and in three Greek families with autosomal dominant inheritance of early-onset L-DOPA-responsive PD[93]. In the affected pedigree, 85% of those carrying the mutation showed clinical PD, whereas it was not seen in any of the 314 controls. Although the α-synuclein A209G (A53T) mutation has not been found in other cohorts of cases with idiopathic PD[5], including in index cases where there is a family history of the disease, another point mutation from G to C has been identified at position 88 of the coding region in exon 3 (A30P) of α-synuclein in a German family[98].

A second gene, *parkin*, has been identified from linkage analysis to map to the long arm of chromosome 6q25.2–27, followed by cloning and sequencing, in a large Japanese family with inherited PD with juvenile onset and a recessive mode of inheritance[94]. This gene is large, involving 500 kilobases with 12 exons. Several patients from three unrelated Japanese families were found to have a deletion of exon 4, and in one patient the deletion involved several (3–7) exons. Parkin is expressed abundantly in the brain, including the substantia nigra, but its cellular and subcellular localization has not been described. Notably, although the syndrome involves a Parkinson-like disorder and the post-mortem pathology in affected cases included degeneration in the substantia nigra and locus coeruleus, Lewy bodies are not a characteristic feature of this variant.

Two further genes have been identified. A mutation in the gene for a thiol protease UCH-L1, involved in the ubiquitin pathway, has been identified in affected members of a small German pedigree[95], indicating a disturbance in the proteolytic pathway resulting in protein aggregation and ubiquitination of α-synuclein in Lewy bodies. The fourth gene is also localized on chromosome 4[96]. In this family there is strong inheritance of pathologically confirmed Lewy-body PD, or PD with dementia, which on genome screen was associated with a chromosome 4p haplotype that segregates with the disease. However, the gene defect is not necessarily expressed as Lewy-body parkinsonism in all carriers, but may alternatively be expressed as an essential tremor.

Further genes for familial PD with linkage to other chromosomal markers have also been suggested, including one designated PARK3 with linkage to markers on chromosome 2p13 (ref. 97), but none of these has yet been identified and sequenced[5].

α-synuclein as a putative pathogenic agent may allow the development of improved animal models based on transgenic technology. Such transgenics are now being created in several laboratories, although as yet no published data are available.

The L-DOPA era

Lewy-body pathology is associated with atrophy and loss of neurons, in particular in the pars compacta of the substantia nigra. This midbrain nucleus is the source of the dopaminergic projection innervating the major motor-control centre of the forebrain, the striatum. In classical pharmacological experiments in the late 1950s, Arvid Carlsson showed that depletion of forebrain dopamine induced akinesia in experimental animals, and he proposed that the motor symptoms of PD might be due to dopamine dysfunction. Hornykiewicz went on to demonstrate a profound loss of dopamine in the striatum of PD patients, soon followed by the first attempts to alleviate symptoms by intravenous administration of the dopamine precursor L-DOPA[12].

The entry of L-DOPA into clinical practice required the efficacy of high doses of oral L-DOPA to be demonstrated, and disabling side effects to be controlled by co-administration of a peripheral DOPA-decarboxylase inhibitor. Combined L-DOPA/carbidopa medication in still the mainstay of symptomatic treatment, and is particularly effective for alleviation of akinesia and rigidity in early- and middle-stage PD. Newer drug developments involve other manipulations of striatal dopamine function by direct-acting dopamine agonists, slow-release L-DOPA formulations, inhibitors of the degrading enzymes catechol-O-methyltransferase (COMT) and monoamine oxidase B (MAO-B), and dopamine transport blockers[12].

Although dopaminergic drugs are very effective during the early stages of PD, their efficacy generally declines as the disease progresses. After several years of treatment with L-DOPA, many PD patients develop severe side effects, most notably dyskinesias at peak dose and 'on–off' fluctuations in drug effectiveness[12,13], and may therefore be forced to reduce the dosage at a time when they need increased antiparkinsonian mediation. These problems have spurred renewed interest in neurosurgical and other interventions that might arrest, reverse or repair the progress of the disease.

Neurosurgical approaches to treatment

Before L-DOPA came into use, a variety of surgical approaches were explored. The pioneering studies by Meyers of stereotaxic lesions in the caudate nucleus and ansa lenticularis led to the identification of the thalamic outputs of the basal ganglia as effective lesion targets ('thalamotomy') for the treatment of tremor. In the absence of modern imaging techniques, these surgical interventions were not particularly reliable. Moreover, although tremor could be controlled, it became apparent that for many PD patients the major functional disability was the akinetic symptoms, which thalamotomy did not alleviate[14]. The recent revival of this approach has derived from three converging factors.

First, improved understanding of the functional organization of the basal ganglia and the functional changes induced by destruction of the nigral dopamine neurons has provided important clues for the identification of critical targets for neurosurgical intervention, in particular the internal segment of the globus pallidus and the subthalamic nucleus[15,16].

Second, several important technical developments now allow surgery to be far more accurate in its selection of targets. Magnetic resonance imaging (MRI) and electrophysiological monitoring during surgery now allow more precise positioning in deep-brain sites. Indeed, in certain targets recording can be combined with stimulation-induced movement to confirm the target site functionally[16].

Finally, the introduction of chronic deep-brain stimulation as an alternative to destructive lesions has provided a new approach for the control of symptoms in advanced PD. High-frequency stimulation seems to produce a functional lesion in the target area, perhaps by depolarization block. Deep-brain stimulation was first introduced as an alternative to thalamotomy for the control of tremor, but has been applied subsequently to both the globus pallidus and subthalamic nucleus for more general improvement of motor disability[17]. It has the dual advantages that the stimulation parameters can be titrated to yield an optimum response over

time, and that it is reversible if adverse effects occur.

One of the main applications of neurosurgery now is not primary symptom relief, but the control of L-DOPA-induced dyskinesia by lesions targeted on the posteroventral globus pallidus (so-called pallidotomy), thereby allowing more effective pharmacotherapy to be extended to the most advanced patients[14].

Mechanisms of cell death in substantia nigra

There is a gradual decline in neurons of the substantia nigra pars compacta and dopamine content of the basal ganglia with age, even in the general population. In idiopathic PD, symptoms become apparent when about 70–80% of striatal dopamine[18] and about 50% of nigral dopamine neurons are lost[19,20]. Conceptually, PD might progress in one of two ways (Fig. 2a). There may be an accelerated rate of cell death, so that the critical level of dopamine loss is reached during the normal life-span. Alternatively, as inspired by the discovery of MPTP toxicity and the environmental toxin theory, an acute insult or stress may cause rapid but partial loss of substantia nigra neurons, followed by continuing decline at the normal age-dependent rate of attrition[21].

Post-mortem analyses have estimated the rate of cell loss in the substantia nigra during normal ageing to be about 5% per decade (Fig. 2b)[19,20]. Nigral cell counts in PD are reduced by about 50% at the onset of symptoms, compared with age-matched controls (and by about 2/3 compared with young individuals, Fig. 2a), and as the disease progresses there is a rapid exponential progression of cell death at a tenfold higher rate (45% per decade; Fig. 2e)[19,20]. Working backwards at the same rate of cell loss, it has been estimated that PD patients would diverge from control baseline about 5 years before the onset of symptoms. The rate of decline of striatal dopamine function, as assessed by serial PET or single-photon emission computed homography (SPECT) scans is even higher (up to 12% per year), but these studies have yielded similar estimates of approximately 4–5 years of disease duration before symptom onset (Fig. 2d)[7].

These data indicate that idiopathic PD may involve not a 'double hit' comprising an acute toxic assault in combination with natural age-related cell loss (brown line in Fig. 2a), but an active disease process in which nigral cell death is accelerated. In addition, the disease is not a slow underlying decline that may have been going on for decades before symptom appearance (the 'accelerated ageing' model, red line) but rather begins in middle age, considerably later than previously suspected, and becomes apparent relatively rapidly (green line).

The mechanisms involved in the progressive degeneration of nigral dopamine neurons in PD are the subject of intense study[22]. In recent years particular attention has focused on several conceptually distinct mechanisms—oxidative stress, mitochondrial dysfunction, excitotoxicity, calcium imbalance, inflammatory changes and apoptosis—which may all interact and amplify each other in a vicious cycle of toxicity leading to neuronal dysfunction, atrophy and finally cell death (Fig. 3).

Oxidative stress. The generation of reactive oxygen species is part of normal cellular metabolism, and cells have evolved a variety of mechanisms for scavenging free radicals[23–25]. These include the conversion of superoxide ions to hydrogen peroxide under the control of the enzyme superoxide dismutase, and the reaction of hydrogen peroxide with reduced glutathione to produce water, under the control of glutathione peroxidase (Fig. 4, equations (2) and (5), respectively).

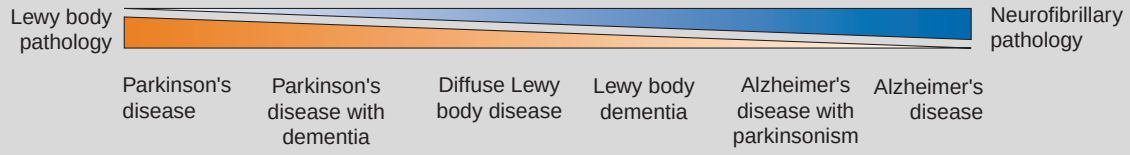
Post-mortem studies in the PD brain indicate that dopamine neurons may be deficient in handling oxidative stress[24,25]. This is believed to reflect both increased generation of free radicals and impairments in the mechanisms for scavenging them: a 30–60% decrease in reduced glutathione in the PD nigra, in conjunction with increased levels of iron associated with the neuromelanin granules, results in a shift in the balance of the capacity to scavenge H_2O_2, generating toxic levels of the hydroxyl ion by the so-called Fenton reaction (Fig. 4, equations (2) and (3)). Indeed, dopamine neurons may be selectively susceptible to the toxic effects of free radicals, as the dopamine molecules themselves contribute to increased free-radical production both by auto-oxidation and through normal enzymatic processing by MAO-B (Fig. 4, equations (1) and (4)). In addition, normal nigral neurons may be susceptible to free-radical toxicity if excessive dopamine is released, as for example under sustained stimulation by methamphetamine.

Mitochondrial dysfunction. The mitochondrion is the primary site for the generation of cellular energy, regulated by five respiratory-

Box 2 Is PD a distinct clinical entity or part of a spectrum of neurodegenerative disorders?

PD and Alzheimer's disease (AD) are generally considered to be separate and distinct clinical entities, with PD involving predominantly motor symptoms associated with loss of dopamine neurons and Lewy-body pathology in the substantia nigra, and AD characterized by manifest cognitive symptoms associated with neurofibrillary tangles (tau) and senile plaques (amyloid-β protein), and primarily affecting cortical areas of the forebrain (see review by Selkoe, this supplement). Nevertheless, there has been increasing recognition that the two diseases overlap[4]. Thus, a substantial proportion of PD patients show cognitive impairments, particularly on tasks of executive function, similar to those seen after frontal-lobe damage[99]. The incidence of dementia in PD patients is 6–12 times higher than in age-matched controls, and most of these (about 75%) have cortical pathology characteristic of AD. Conversely, about two-thirds of diagnosed AD patients develop extrapyramidal symptoms, such as bradykinesia and rigidity, and many have neurodegenerative changes in the substantia nigra consistent with the diagnosis of PD. The extent of overlap between the two diseases is thus much greater than would be expected to occur by chance[4].

Consequently, we need to consider whether the two diseases may not be distinct entities, as generally considered, but rather be extremes of a spectrum of disease. Perl and colleagues[4] propose that PD and AD should be viewed as part of a continuum of neurodegenerative disorders with considerable overlap (see inset). Patients with similar pathology, involving both Lewy bodies in the substantia nigra and senile plaques and neurofibrillary tangles in the neocortex, are as likely to be diagnosed as 'AD with parkinsonism' as 'PD with dementia'. Alternatively, if both classes of symptom are apparent at first appearance, the diagnosis may be 'diffuse Lewy-body disease' or 'Lewy-body dementia'[4]. This emerging concept suggests that multiple aetiologies can lead to similar clinical phenotypes, and that there may exist unifying neurodegenerative mechanism(s), triggered by different aetiological factors, that are expressed differently in different disease entities. Thus, in future research on pathogenesis and treatment, it may be important to consider not only overlaps between the major neurodegenerative diseases, but also heterogeneous aetiologies within each disease entity.



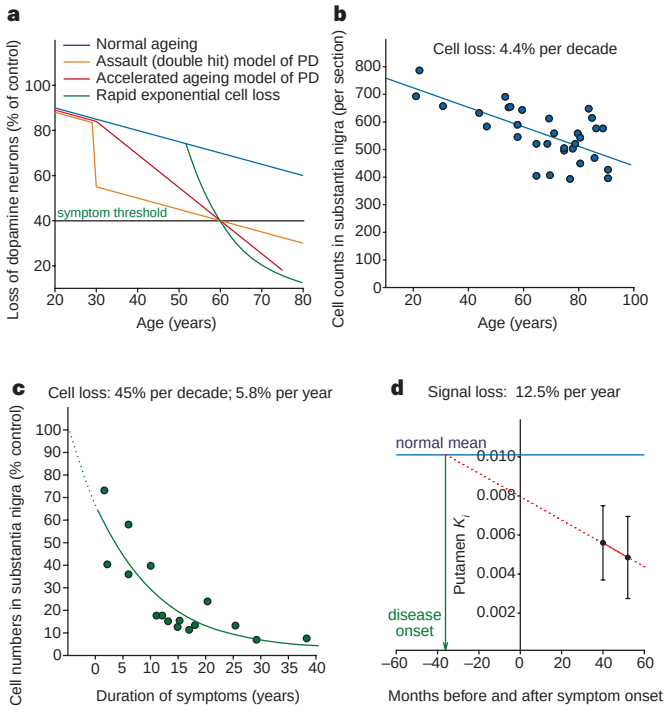


Figure 2 Rate of progression in Parkinson's disease. **a**, Models of disease progression. Normal age-dependent cell loss in the substantia nigra is shown in blue. PD symptoms become apparent after a critical level of cell loss, the 'symptom threshold'. In the 'accelerated ageing' model of PD (red), cell loss starts early and progresses more rapidly, whereas in the 'double hit' or 'assault' model (brown) there is subthreshold cell loss resulting from acute toxic insult, followed by a normal rate of age-dependent cell loss until the critical threshold is reached. Recent evidence favours an even more rapid exponential cell loss, departing from normal numbers only a few years before symptom onset (green). **b**, The rate of nigral cell loss in a normal population determined from post-mortem studies is ~4.4% per decade (from ref. 19). **c**, Parallel studies in PD suggest a tenfold higher rate of cell loss[19] (from ref. 19). **d**, An even greater rate of decline is concluded from estimates based on serial measurements of fluorodopa binding constants by PET (from ref. 91). These data support the view that PD involves an active process of cell death with an onset 4–5 years before symptoms appear (dashed lines in **c**, **d**).

chain complexes. Complex I controls the transfer of one electron from NADH to co-enzyme Q and the transfer of two protons to the mitochondrial inter-membrane space, which are then used by complex V to synthesize ATP from ADP, the main energy supply of the cell. The toxicity of MPP+, the active ion of MPTP, seems to result from its inhibition of mitochondrial respiration at the level of complex I, resulting in a rapid fall in ATP levels and eventual cell death due to energy failure[23]. Moreover, MPP+ can increase leakage of electrons at complex I, thereby increasing mitochondrial generation of superoxide.

Identification of the site of MPTP cytoxicity has naturally led to investigation of mitochondrial function in PD. Complex I activity is decreased in the substantia nigra[26], an effect which is specific to PD, and not seen in other diseases affecting the same neurons. However, there is typically only a moderate decline in complex I activity, which cannot alone readily account for the extent of cell death in this nucleus[23]. Nevertheless, mitochondrial dysfunction may contribute to a cascade of changes, including a reduction of ATP supplies for other cellular processes, reduced efficiency of sodium and calcium pumps, raised intracellular calcium and increased oxidative stress, thus contributing to the vicious circle leading to cell death.

Excitotoxic damage. Damage due to excess glutamate, which changes the permeability of cells to calcium by acting at NMDA (*N*-methyl-D-aspartate) receptors, is considered to be a major

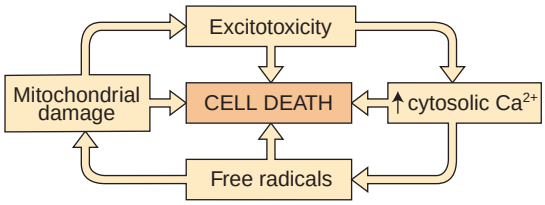


Figure 3 Oxidative stress, mitochondrial dysfunction, excitotoxicity and calcium influx each contribute to cell death, and enhance each other in a cycle of toxicity[92].

mechanism of neurodegeneration in ischaemia, and similar processes are also believed to operate in PD. In particular, the substantia nigra receives rich glutamatergic inputs from both the neocortex and subthalamic nucleus. Activation of NMDA receptors allows an influx of calcium followed by activation of nitric oxide synthase (NOS), increasing the generation of toxic free radicals through the peroxynitrite reaction (Fig. 4, equation (6)).

Cell death. When induced by the toxins 6-hydroxydopamine (6-OHDA) or MPTP in animal models of PD, nigral cell death seems to involve both nectoric and apoptotic processes. In human PD there has been some debate about whether key features of apoptosis could be demonstrated, at least when based on morphological features or TUNEL assays alone[27,28]. However, the recent development of techniques involving double labelling with TUNEL to demonstrate DNA fragmentation in conjunction with a cyanine dye that binds to DNA to provide structural detail has demonstrated chromatin condensation and DNA fragmentation within the same nuclei of nigral dopamine neurons in PD[28]. These results indicate that the number of apoptotic nuclei in the substantia nigra in PD is greater than that seen in normal ageing, consistent with the 10-fold-higher rate of cell loss seen in patients with disease[3,29].

Neuroprotective therapies

The progressive nature of PD and the fact that neuronal degeneration in the substantia nigra is slow and protracted present opportunities for therapeutic intervention aimed at blocking or slowing down the degenerative process. Recent neuroimaging and autopsy data (see above) indicate that there is a preclinical period of 4–5 years before symptoms appear, and that the rate of cell loss and decline of dopaminergic function in the striatum is likely to be in the order of 10% per year, with the disease progressing relatively more rapidly during the early phases than in the more advanced stages of the disease. Both PET and SPECT imaging seem to be able to detect a decline in striatal dopamine function before clinical symptoms appear[7], which may make it possible to begin neuroprotective intervention during the preclinical phase.

The neurodegenerative process in PD is likely to involve a cascade of inter-related events—oxidative stress, mitochondrial dysfunction, excitotoxicity with excess formation of ·NO and O_2^-, and inflammatory changes, leading to both apoptotic and necrotic cell death (Fig. 3)—each of which offer potential targets for neuroprotective therapeutic strategies. Because the dopamine neurons are probably dysfunctional for some time—possibly years—before they are irreversibly damaged, both neurotrophic and antiapoptotic agents may be used to prevent delayed cell death and/or restore function.

Antioxidants. The largest neuroprotective clinical trial conducted to date, the DATATOP study, involved two putative antioxidative agents, vitamin E and deprenyl (selegiline)[30]. Vitamin E had no significant effect at the doses used, but deprenyl slowed the early progression of symptoms and delayed the emergence of disability by an average of nine months. However, being an MAO-B inhibitor, this drug has symptomatic effects of its own, which has confounded interpretation of the results[30]. Interestingly, animal studies have suggested that the neuroprotective effect is not dependent on MAO-B inhibition *per se*, but rather on an antiapoptotic effect of the

metabolite desmethyl-deprenyl, possibly acting on protein transcription[29,31]. There are many alternative antioxidative approaches that may be considered in future clinical trials, including free-radical scavengers, glutathione-enhancing agents, ion chelators and drugs that interfere with the oxidative metabolism of dopamine. Interestingly, the classic directly acting dopamine-receptor agonists may belong to the last group: by stimulating dopamine autoreceptors, these drugs reduce dopamine synthesis, turnover and release, so that less L-DOPA is needed. In addition, some of these compounds have direct antioxidant effects[32]. Clinical trials specifically designed to test the neuroprotective effects of dopamine agonist treatment in early PD are now underway in several centres.

Excitotoxicity. The renewed interest in excitotoxic mechanisms in PD has been stimulated by the finding that the output neurons of the subthalamic nucleus provide a powerful glutamatergic excitatory input to the substantia nigra, and that these neurons are hyperactive in animals with lesions of the nigral dopamine system. Increased glutamate release may increase Ca^{2+} influx into the cells and increase formation of nitric oxide by activating nitric oxide synthase (NOS) (Fig. 4, equation (6)). This may be particularly harmful in PD because the defect in mitochondrial complex I may make the dopamine neurons vulnerable even to physiological concentrations of glutamate[33]. In support of this toxicity mechanism, both glutamate-receptor blockers and neuronal NOS inhibitors have been reported to attenuate degeneration of dopamine neurons in MPTP-treated monkeys[33]. The specificity of this effect is discussed in detail in ref. 3. Thus, excitotoxic damage to the substantia nigra may, at least in part, depend on the integrity of the subthalamic nucleus; inactivation of this nucleus by deep brain stimulation (see above) may therefore also have a neuroprotective effect. Although there is no clinical evidence in support of this mechanism, lesions of the subthalamus may protect the nigral dopamine neurons in the 6-OHDA-lesioned rat[33]. Other treatment strategies aimed at reducing subthalamic overactivity may include dopamine agonists and glutamate-receptor antagonists, the first of which, remacemide, is now in clinical trials[3].

Neurotrophic and antiapoptotic factors. Neurotrophic factors were originally identified as target-derived compounds that regulate neuronal survival and growth during embryonic development. However, these factors also have neurotrophic effects in the mature nervous system and can rescue injured neurons after toxic, mechanical or ischaemic damage in the adult brain. A wide range of factors can protect nigral dopamine neurons *in vivo* in animal models of PD, notably glial cell line-derived neurotrophic factor (GDNF), neurturin, basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), neurotrophins 3 and 4/5, ciliary neurotrophic factor and transforming growth factor-β[34,35].

The GDNF family of proteins are particular interesting because of their potent *in vivo* effects[36]. In the standard 6-OHDA and MPTP lesion models, recombinant GDNF has three different effects on dopamine neurons: (1) direct rescue of injured or axotomized neurons when given before or shortly after the insult; (2) promotion of axonal sprouting or regeneration in chronically lesioned animals; and (3) stimulation of dopamine turnover and function in lesioned, and possibly also intact, nigral neurons[36,37]. However, the mechanisms by which exogenous GDNF—whether administered intracerebrally or into the ventricular system—induces symptomatic relief in behaviourally impaired animals is poorly understood. Nevertheless, clinical trials using intraventricular injections of GDNF have begun[38].

Alternative neurotrophic agents include the so-called immunophilin ligands, which have neurite-growth-promoting and neuroprotective effects *in vitro* on many neuronal cell types, including

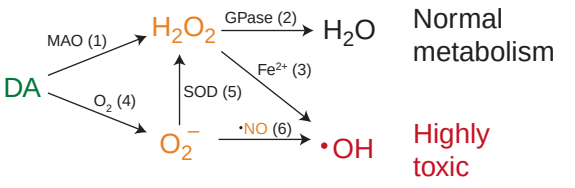


Figure 4 Free radicals in Parkinson's disease. Excess generation of free radicals may constitute one of the major pathogenic processes in PD[25]. Although it is part of normal metabolism in all cells, free-radical generation is particularly linked to dopamine (DA) metabolism, as illustrated here, by either metabolic conversion (through 3,4-dihydroxyphenyl-acetaldehyde (3,4-DOPAA) to DOPAC; equation (1)) or auto-oxidation (equation (4)). Whereas metabolism through H_2O_2 to H_2O (equations (2) and (5)) neutralizes toxicity, superoxide (O_2^-) and nitric oxide (·NO) are themselves moderately reactive, and metabolism through the Fenton and peroxynitrite ($ONOO^-$) reactions (equations (3) and (6), respectively) yield the highly toxic hydroxyl ion (·OH)[3]. The most toxic ions are indicated in bold type.

$$DA + O_2 + H_2O \xrightarrow{MAO\text{-}B} 3,4\text{-DOPAA} + NH_3 + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{reduced glutathione} \xrightarrow{\text{glutathione peroxidase}} \text{oxidized glutathione} + 2H_2O \quad (2)$$

$$H_2O_2 \xrightarrow{Fe^{2+} \rightarrow Fe^{3+}} \mathbf{\cdot OH} + OH^- \quad (3)$$

$$DA + O_2 \longrightarrow \cdot SQ + O_2^- + H^+$$
$$DA + O_2^- + 2H^+ \longrightarrow \cdot SQ + H_2O_2 \quad (4)$$

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (5)$$

$$O_2^- + \cdot NO \longrightarrow \mathbf{ONOO^-} \rightarrow \mathbf{\cdot OH} + \cdot NO_2 \quad (6)$$

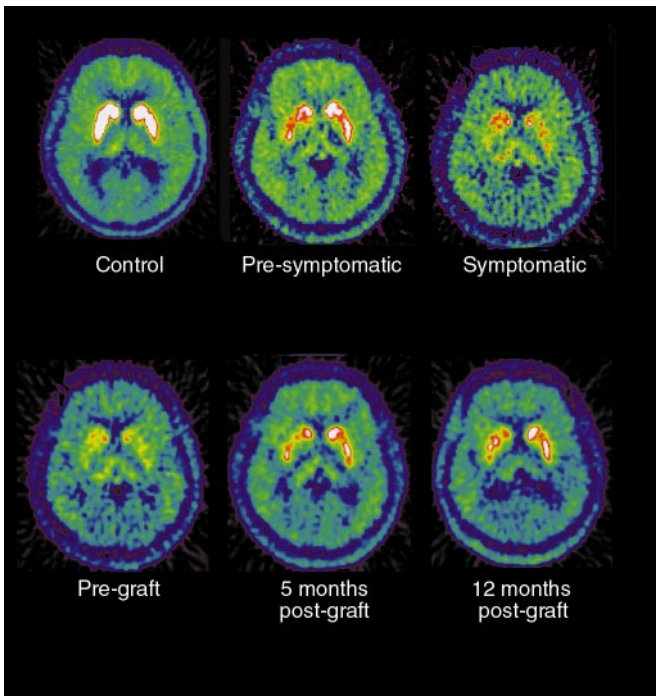


Figure 5 *In vivo* imaging of dopaminergic activity in the Parkinsonian basal ganglia shown by [^{18}F]fluorodopa PET. Top: the signal from striatum in a healthy control subject, a patient with symptomatic Parkinson's disease and a twin who was asymptomatic at the time of scan but who subsequently developed the disease. Bottom: a patient with advanced PD, before and at 5 and 12 months after transplantation of embryonic nigral tissue into the striatum bilaterally. Note the progressive increase in fluorodopa signal over time as the transplant matures. (Images supplied by P. Piccini and D. Brooks, MRC Cyclotron Unit, Hammersmith Hospital, UK.)

mesencephalic dopamine neurons. These neurotrophic effects were initially observed in experiments with the immunosuppressive drugs, cyclosporin A and FK506. However, non-immunosuppressive analogues, which are thought to act by a different mechanism (regulation of intracellular Ca^{2+} release), have shown that the neurotrophic effect can be dissociated from the immunosuppressive one[39]. Initial experiments in various lesion models, including 6-OHDA- and MPTP-lesioned rats and mice[40,41], indicate that systemically administered low-molecular-mass compounds may be promising therapeutic agents in neurodegenerative disease.

New insight into the intracellular events leading to apoptosis in dopamine neurons, particularly the role of mitochondria, may provide additional potential therapeutic targets. Decreased amounts of mitochondrial complex I in PD (see above) have been proposed to make the dopamine neurons more vulnerable to the sequence of events that underly the cell-killing mechanism that involves opening of the so-called mitochondrial megachannel[42]. Thus, drugs that act at the mitochondrial stage of apoptosis (for example, by maintaining closure of the megachannel), or further downstream (for example, by blocking the release of apoptosis-inducing factors or inhibiting caspase enzymes[43]), may be interesting therapeutic candidates[29,42].

Cell replacement therapies

Neural transplantation. Neural grafting in PD is based on the idea that dopamine supplied from cells implanted into the striatum can substitute for the lost nigrostriatal neurons. In rodent and primate models of PD, embryonic dopamine neurons grafted to the denervated striatum can establish a functional innervation and restore dopaminergic neurotransmission in the area surrounding the transplant[44]. However, to survive transplantation, dopamine neurons must be obtained at an early stage of development, and they must be placed ectopically (in the striatum rather than in the substantia nigra) to connect up with the dopamine-receptor-bearing target cells and exert their functional effects.

Clinical trials have shown that mesencephalic dopamine neurons obtained from human embryo cadavers can survive and function in the brains of patients with PD[45,46]. PET scans have shown significant increases in [^{18}F]fluorodopa uptake in the area around the graft that has been maintained for at least six years in several patients[47] (Fig. 5). Long-lasting symptomatic improvement has been reported in a majority of the grafted patients, and in the most successful cases it has been possible to withdraw L-DOPA treatment[45,46]. However, functional recovery has so far only been partial, and both the efficacy and reproducibility of the procedure must be improved.

Two cases that have come to autopsy have shown good survival of grafted dopamine neurons and extensive axonal outgrowth into the grafted putamen[48]. Immunological rejection of the human-to-human allografts has not been reported in any PD patient, even several years after withdrawal of immunosuppressive treatment[47]. However, abundant macrophages and T and B lymphocytes were observed in otherwise healthy-looking and functional transplants in the two autopsy cases, 12 months after withdrawal of cyclosporin immunosuppression (18 months after transplantation)[49], indicating the potential for a host-derived immune response in intracerebral allografts when immunosuppression is removed.

The demonstration that embryonic dopamine neurons can survive and function in the human brain represents a first important step towards a cell replacement therapy in PD. Current research is aimed at improving the survival and growth of transplanted dopamine neurons, and finding alternative sources of cells for grafting. The main limitations of current cell-transplant procedures are the ethical, practical and safety issues associated with tissue derived from aborted human fetuses[50], and the large amounts of embryonic mesencephalic tissue that are needed to obtain therapeutic effects in patients, which severely restricts the possibility of applying this procedure outside highly specialized centres.

In current grafting protocols, no more than 5–20% of the expected numbers of grafted dopamine neurons survive. Consequently, tissue from at least 3–4 embryos, yielding about 100,000–150,000 surviving dopamine neurons, needs to be implanted on each side of the patient's brain to induce significant therapeutic improvement[45,51]. Addition of free-radical scavengers, caspase inhibitors or neurotrophic factors to the fetal cell preparation may increase dopamine neuron survival 2–3 fold[43,52]. Moreover, in the rat PD model, administration *in vivo* of neurotrophic factors (including GDNF, BDNF and bFGF) to the transplants during the first weeks after implantation enhances both survival and growth of intrastriatal dopamine neuron transplants[53–55]. Application of these principles to clinical protocols may reduce the need for multiple donor embryos and increase the functional efficacy of the grafted cells.

The ethical issues surrounding the use of human embryonic cells for grafting remains a matter of concern[50]. Cells from other species may offer a solution. Cells transplanted between species survive well in the brain, which is partly protected from the body's immune system, provided the recipient is treated with immunosuppressive drugs[56]. Indeed, clinical trials using porcine cells are already underway in both PD and Huntington's disease patients[57,58]. However, the use of xenotransplants in humans remains controversial, not least because effective and acceptable techniques for long-term immunosuppression across the species barrier are not yet fully established. Moreover, there remain concerns about a theoretical risk of cross-species transfer of infectious agents, in particular animal retroviruses[57,59].

Expansion of neuronal progenitors. Until now, transplantation of dopamine neurons has focused primarily on differentiated neuroblasts and young postmitotic neurons, at the stage of neuronal development which has been found empirically to be optimal for survival, growth and establishment of functional connectivity of the explanted cells. However, precursor cells taken at earlier stages of development, when they are in an active proliferative phase, might prove more effective. Thus, if it were possible to expand precursor cells *in vitro* and control their terminal differentiation into mature dopamine neurons, then large numbers of cells could be expanded and made available for transplantation as required. This would have the additional advantage that such cells can be standardized, screened and manipulated (for example, by cell sorting or gene transduction) in ways that could never be possible with the limited quantities of fresh tissue that are available.

It is now possible to expand the precursors of mesencephalic dopamine neurons *in vitro* by stimulation with high concentrations of growth factors (typically bFGF and/or EGF), and expanded cells can subsequently be induced to differentiate into mature dopamine neurons[60–63]. The expanded cells can survive and function after transplantation to the striatum in the rat PD model[62], although the overall yield of surviving dopamine neurons *in vivo* was quite low owing to substantial loss of cells (95–98%) in the grafting step. If neural progenitors are to be used for neuronal replacement in PD, it will be essential to control the steps leading to induction of a dopaminergic phenotype. This is likely to be a complex process involving temporally and spatially controlled cell–cell interactions and specific signalling molecules. Indeed, several factors likely to be important in this process have been identified, including sonic hedgehog, Nurr1 and FGF-8 in particular[64].

Alternative sources of cells. It is at present unclear whether cells used for grafting in PD must be neuronal (that is, exhibit impulse-dependent release of dopamine at synaptic sites) or whether non-neuronal cells that secrete dopamine (or its precursor L-DOPA) constitutively in a diffuse, non-synaptic manner would suffice. Adrenal chromaffin cells were the first cells of the latter type to be investigated. These cells, which produce dopamine and several other catecholamines, as well as a variety of neuroactive and neurotrophic factors, have the advantage that they can be obtained from one of the patient's own adrenals. However, the long-term survival of chromaffin cells is very poor in the brain, and the initial functional effects that are seen after implantation into the striatum (which are

unlikely to be due to dopamine secretion alone) are not well sustained at longer survival times, either in experimental PD or in PD patients[65,66]. Owing to the poor long-term therapeutic benefit and considerable morbidity associated with intracerebral transplantation of adrenal medullary tissue, this approach is no longer actively pursued clinically.

These problems have stimulated the search for cells that survive better and can sustain considerably higher levels of *in vivo* dopamine release after transplantation to the brain. Experiments using implants of dopamine-releasing polymers[67,68] or encapsulated dopamine- and L-DOPA-producing PC12 cells[69,70] have shown that implants that act as a 'biological minipump' can tonically activate dopamine receptors and induce limited functional effects in the denervated striatum, without any synaptic connections. Working on the same principle, primary non-neuronal cells producing dopamine, such as glomus cells from the carotid body[71], and cells engineered to produce high levels of L-DOPA and/or dopamine[72–74] are being investigated (see below). However, the only functional responses that have been obtained with these kinds of cell (normalization of dopamine-receptor supersensitivity as reflected in a reduced motor response in the standard rotation test) involve a pharmacological mechanism of action, and it remains to be seen whether dopamine-secreting cells can provide any long-lasting improvements in more complex aspects of the motor symptoms in rat or monkey PD models, or indeed in human PD. Nevertheless, as clinical observations indicate that continuous L-DOPA delivery might be advantageous in PD[75], it seems reasonable that cells engineered to provide a constant local source of L-DOPA might prove at least as efficient as standard systemic L-DOPA therapy.

Gene transfer approaches

Gene transfer in PD could be used in two ways: to replace dopamine in the affected striatum by introducing the enzymes responsible for L-DOPA or dopamine synthesis; and to introduce potential neuroprotective molecules that may either prevent the dopamine neurons from dying or stimulate regeneration and functional recovery in the damaged nigrostriatal system.

In vivo, dopamine is synthesized from tyrosine by two enzymes, tyrosine hydroxylase and aromatic-amino-acid decarboxylase (AADC). However, only tyrosine hydroxylase is rate limiting, and AADC is present at sufficient levels even in the dopamine-denervated striatum, within non-dopaminergic neurons and glial cells. The current strategy, therefore, is to express tyrosine hydroxylase alone, either in striatal cells by direct gene transfer *in vivo*, or by transduction of cells in culture, *ex vivo*, before transplanting them to the striatum[76,77]. Functional activity of tyrosine hydroxylase depends on the availability of its cofactor, BH_4, and as the level of the cofactor may be insufficient in the denervated striatum it is likely that both tyrosine hydroxylase and the BH_4-synthesizing enzyme, GTP cyclohydrolase 1, will have to be transduced to obtain sufficient levels of L-DOPA production *in vivo*[78,79].

Although positive results have been reported using this approach, the functional effects have not been long-lasting. Tyrosine hydroxylase is an unstable protein in non-catecholaminergic cells, which may explain why long-term expression of high levels of tyrosine hydroxylase and L-DOPA production has not yet been achieved in the rat PD model[77]. Moreover, most *in vivo* studies to date have used the unilateral 6-OHDA-lesioned rat model, with apomorphine-induced rotation as the functional assay. Reduced apomorphine-induced rotation, which has been reported in a number of studies using either *in vivo* or *ex vivo* transfer of the tyrosine hydroxylase gene[76,77], is not a reliable functional indicator of the degree of symptomatic improvement or the extent of recovery of striatal L-DOPA or dopamine synthesis[80,81]. Thus, it remains unclear whether local intrastriatal delivery of L-DOPA or dopamine from genetically transduced cells, or from direct *in vivo* tyrosine hydroxylase gene delivery, can produce any substantial symptomatic improvement in experimental PD.

Attempts to use gene transfer to block degeneration in the nigrostriatal system have so far been focused on neurotrophic factors, in particular BDNF and GDNF. Promising results have been obtained in the rat PD model with transplants of fibroblasts or fibroblast cell lines engineered to secrete BDNF[82,83] or GDNF[84], and with injections of adenovirus or adeno-associated virus vectors carrying the GDNF gene administered close to the substantia nigra[85,86] or into the striatum[87,88]. Interestingly, in these experiments, nanogram amounts of the factors have typically been measured from the transduced cells, and the neuroprotective effects have been in the order of 40–70% rescue of nigral dopamine neurons. This contrasts with the microgram amounts required to achieve a similar magnitude of protection after intracerebral injection or infusion of recombinant GDNF or BDNF indicating that intracerebral gene transfer is an efficient way to obtain sustained activation of neurotrophin receptors in the brain.

In these experiments, gene transfer was undertaken before injection of 6-OHDA or MPTP, and the observed effects may thus be seen as protection against an acute toxic insult. This may not provide a relevant model for the slow, progressive degenerative process in PD. In the acute rat model, short-term delivery over a couple of weeks, limited to a small brain region, may be sufficient, whereas long-term expression of the factor, probably over several years and in considerably larger amounts, may be necessary for efficient neuroprotection in the chronic clinical condition. Alternative ways to intervene in the degenerative process may include direct gene transfer of molecules involved in the cell-killing cascade, such as Bcl-x[89] or superoxide dismutase[90]. These approaches, however, have so far not been explored in models of PD.

Conclusions

Recent advances in our understanding of cellular and molecular foundations of PD have led to new perspectives on the disease process and the identification of new pharmacological, neuroprotective and surgical approaches to therapy. Whereas current therapies are primarily targeted at symptomatic relief, recent discoveries relating to the molecular pathology and mechanisms of cell death in PD offer for the first time the prospect of protective therapies aimed at halting and/or reversing the progress of the disease process itself. The rate of progress in this field is impressive, and further major advances in our understanding of the relationship between gene mutations, α-synuclein deposition and aggregation, Lewy-body formation, disturbance of internal cellular metabolism and selective nigrostriatal cell death appear likely within the near future. We believe that it is realistic to expect that, within the next decade, these developments will provide new clinical treatments based on effective protection against the degenerative disease process itself. □

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