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Gene–environment interactions: key to unraveling the mystery of Parkinson’s disease

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Abstract

Parkinson’s disease (PD) is the second most common neurodegenerative disease. The gradual, irreversible loss of dopamine neurons in the substantia nigra is the signature lesion of PD. Clinical symptoms of PD become apparent when 50–60% of nigral dopamine neurons are lost. PD progresses insidiously for 5–7 years (preclinical period) and then continues to worsen even under the symptomatic treatment. To determine what triggers the disease onset and what drives the chronic, self-propelling neurodegenerative process becomes critical and urgent, since lack of such knowledge impedes the discovery of effective treatments to retard PD progression. At present, available therapeutics only temporarily relieve PD symptoms. While the identification of causative gene defects in familial PD uncovers important genetic influences in this disease, the majority of PD cases are sporadic and idiopathic. The current consensus suggests that PD develops from multiple risk factors including aging, genetic predisposition, and environmental exposure. Here, we briefly review research on the genetic and environmental causes of PD. We also summarize very recent genome-wide association studies on risk gene polymorphisms in the emergence of PD. We highlight the new converging evidence on gene–environment interplay in the development of PD with an emphasis on newly developed multiple-hit PD models involving both genetic lesions and environmental triggers.

1. Introduction

Parkinson’s disease (PD), an age-related neurodegenerative movement disorder, affects more than 1 million Americans and 4 million people worldwide at any one time. While 5–10% of cases are considered of early-onset (PD occurring before 50 years of age), the average age of onset of PD is around 60 years. PD patients suffer from resting tremor, bradykinesia (slowed movements), muscle rigidity, and postural instability. These symptoms result from the degeneration of dopamine-containing neurons in the substantia nigra (SN) and the consequent loss of dopamine, a neurotransmitter involved in the fine modulation of motor function. Dopamine replacement therapy (the main treatment for PD) using the metabolic precursor of dopamine, L-DOPA, or dopamine receptor agonists, can temporarily alleviate motor symptoms rather than retard PD progression. This treatment cannot relieve concomitant nonmotor symptoms (e.g. autonomic dysfunction, depression, anxiety and dementia) that also have a high impact on PD patients. Unsuccessful translation of

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encouraging preclinical research to clinical therapeutics pinpoints the urgent need for better approaches and better animal models to unravel mechanisms of PD neurodegeneration and to discover disease-modifying therapies for PD.

Both genetic defects and exposure to environmental risk factors are linked to PD. The occurrence of PD-causative genetic defects in apparently sporadic cases of PD and even in healthy carriers as well as the highly variable onset ages and considerable phenotypic variations in inherited PD underscore a crucial role of gene-environment interactions in the development of PD. The lack of overt loss of nigral dopamine neurons—the signature lesion of PD—in most gene-based PD animal models and the paucity of α -synuclein-containing Lewy body inclusions—the pathological hallmark of PD—in most toxin-based PD models further support the essential role of gene-environment interplay in PD pathogenesis. Newly developed two-hit PD models, created with combinations of genetic lesions and environmental triggers, are invaluable for better defining precise disease mechanisms and screening putative disease-modifying therapeutics. Epidemiological studies aimed at identifying gene-environment interactions in PD development may also prove to be fruitful.

2. The genetic causes of PD

2.1 Familial aggregation and twin studies of PD

Approximately 10–30% of patients with PD report a first-degree relative with parkinsonism (Farrer, 2006; Rocca et al., 2004; Sveinbjornsdottir et al., 2000). Such familial aggregation of PD used to be considered as a consequence of shared exposure to a common environment. PD was traditionally considered a non-genetic disorder of ‘sporadic’ origin, since heritability estimates in twin studies revealed conflicting results that were heavily weighted against a genetic basis for this disease. For instance, multiple studies show low concordance in both monozygotic and dizygotic twins (Duvoisin *et al.*, 1981; Marttila *et al.*, 1988; Tanner, 2003; Ward *et al.*, 1983; Wirdefeldt *et al.*, 2004). However, a higher risk ratio for concordance in monozygotic versus dizygotic twins was reported in early-onset PD patients (Tanner et al., 1999). Striatal ^{18}F -dopa positron emission tomography (PET) scan, a presymptomatic detection marker of PD, show three times higher concordance rate for PD in monozygotic twins (55%) than in dizygotic twins (18%), implying a significant genetic contribution (Piccini et al., 1999). By minimizing methodological problems in ascertainment and recall bias, twin studies can achieve more consistent results and advance our understanding of genetic influence on PD.

2.2 Monogenically inherited PD

Recent advances in molecular genetics have unveiled that 5–20% of PD patients from different geographical areas have monogenic forms of PD. Numerous genetic defects (~82% simple mutations and ~18% copy number variations) in more than 13 loci and 9 genes have been identified in inherited PD patients and families (Dawson *et al.*, 2010; Lesage and Brice, 2009; Nuytemans *et al.*, 2010). To date, a causal role for PD has been confirmed with five genes: *SNCA* (*PARK1*) encoding α -synuclein, *Parkin* (*PARK2*) encoding Parkin, *PINK1* (*PARK6*) encoding PTEN-induced putative kinase 1 (PINK1), *DJ-1* (*PARK7*) encoding DJ-1, and *LRRK2* (*PARK8*) encoding Leucine-rich repeat kinase 2 (LRRK2). Extensive mutation screening of these five causative genes has discovered approximately 330 confirmed or possible pathogenic mutations in over 1900 families (Nuytemans et al., 2010).

2.3 Dominantly inherited mutations

2.3.1 *SNCA* (*PARK1*) and α -synuclein—*SNCA* encodes α -synuclein that consists of 140 amino acids and is a natively unfolded, soluble protein in the cytoplasm or associated with lipid membranes (Davidson et al., 1998). Although physiological functions of α -

synuclein is still not fully understood, experimental evidence implicates α -synuclein in neurotransmitter release, vesicle turnover, synaptic plasticity, and intracellular trafficking within the endoplasmic reticulum/Golgi network (Abeliovich *et al.*, 2000; Chandra *et al.*, 2004; Cooper *et al.*, 2006; Gitler *et al.*, 2008; Liu *et al.*, 2004). A recent elegant genetic study illustrates that α -synuclein directly binds to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-protein synaptobrevin-2/vesicle-associated membrane protein 2 and promotes SNARE-complex assembly in vivo and in vitro. Thus, α -synuclein may function to maintain normal SNARE-complex assembly and participates in repeated neurotransmitter release in presynaptic terminals (Burre *et al.*, 2010).

Three missense mutations in α -synuclein, Ala53Thr, Ala30Pro and Glu46Lys, cause a autosomal dominant PD in rare kindreds (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997; Zarranz *et al.*, 2004). *de novo* genomic multiplications of *SNCA* are linked to familial PD; age of onset as well as duration and severity of disease are gene copy number-dependent (Chartier-Harlin *et al.*, 2004; Farrer *et al.*, 2004; Ibanez *et al.*, 2004; Singleton *et al.*, 2003). Interestingly, *SNCA* duplications were also reported in four apparently sporadic PD patients (Ahn *et al.*, 2008; Nishioka *et al.*, 2009). Common variability within the promoter or 3'-UTR of *SNCA* is associated with increased α -synuclein expression and a greater risk of sporadic PD (Mueller *et al.*, 2005; Pals *et al.*, 2004; Winkler *et al.*, 2007).

Under pathological condition, α -synuclein undergoes conformational changes including oligomerization and fibrillogenesis; unstructured monomeric α -synuclein self-assembles into protofibrils (heterogeneous and metastable assembly intermediates) and mature fibrils (Conway *et al.*, 2000; Conway *et al.*, 2001). While both α -synuclein protofibrils and fibrils are cytotoxic (Lee and Trojanowski, 2006; Volles *et al.*, 2001), some studies suggest that sequestration of protofibrillar intermediates, more toxic form of α -synuclein, into mature fibrils may be protective (Forman *et al.*, 2005; Volles *et al.*, 2001). Filamentous aggregates of misfolded α -synuclein protein are the major components of Lewy bodies in both familial and sporadic PD (Spillantini *et al.*, 1997). α -synuclein carrying missense substitutions often presents reduced affinity for lipids and adopts a propensity for misfolding and accelerated fibril formation (Conway *et al.*, 2000) (Figure 1). The identity of the most toxic α -synuclein species and the better understanding of the structural basis of α -synuclein aggregation and toxicity are crucial for the development of better diagnosis and prognosis tools as well as effective therapeutics for PD. Age-associated increases of α -synuclein in monkeys and humans are related to nigrostriatal dopamine depletion (Chu and Kordower, 2007). The formation of α -synuclein-containing inclusions in grafted fetal mesencephalic neurons in PD patients strongly indicates a prominent role of α -synuclein in PD progression and even host-to-graft propagation (Li *et al.*, 2008; Maries *et al.*, 2003; Mendez *et al.*, 2008).

2.3.2 LRRK2 (PARK8) and LRRK2 (dardarin)—The *LRRK2* transcript contains 51 exons encoding for a 2527 amino acid protein, LRRK2 (also known as Dardarin). Several predicted functional domains (e.g. Roc GTPase, Roc-COR tandem domain and protein kinase domains) in LRRK2 suggest this protein have multiple functions, including protein–protein interactions (scaffold protein function), substrate binding, and protein phosphorylation (Deng *et al.*, 2008b; Liu *et al.*; Mata *et al.*, 2006; Xiong *et al.*, 2010). Under physiological conditions, LRRK2 is found to form dimers (Greggio *et al.*, 2008) and may play a role in neuronal outgrowth and guidance (MacLeod *et al.*, 2006; Sakaguchi-Nakashima *et al.*, 2007). Wildtype LRRK2, but not its mutants, attenuates oxidative stress-induced cell death, indicating a protective role for LRRK2 (Liou *et al.*, 2008). A recent study using LRRK2 knockout mice hints at an essential role of LRRK2 during aging in the regulation of protein homeostasis and, specifically, α -synuclein through the regulation of protein degradation pathways. Although the dopaminergic system of LRRK2 null mice appears normal, the loss of LRRK2 in kidneys of aged mice leads to impairment of

autophagy-lysosomal pathways, age-dependent accumulation and aggregation of α -synuclein (60-fold) and ubiquitinated proteins, inflammatory responses, oxidative damage, and apoptotic cell death (Tong et al., 2010). Moreover, LRRK2 has been reported to interact with the microRNA pathway to regulate protein synthesis in *Drosophila*, and pathogenic LRRK2 carrying I2020T or G2019S mutations suppresses microRNA-mediated translational repression of E2F1/DP, transcription factors implicated in cell cycle and survival control (Gehrke et al., 2010). However, biological functions, physiological substrates, binding partners, and regulators of kinase activity of LRRK2 merit further exploration.

Mutations in the *LRRK2* gene, the most common known cause of PD, are associated with autosomal dominant and sporadic forms of PD (Correia Guedes et al., 2010; Di Fonzo et al., 2005; Farrer, 2006; Nichols et al., 2005; Paisan-Ruiz et al., 2004). More than 80 missense mutations identified so far spread across the entire LRRK2 protein and affect all predicted functional domains. The clinical manifestations of homozygous carriers seem not different from those of heterozygous carriers (Lesage et al., 2005). Unlike other PD-causative genes, mutations in *LRRK2* have been detected in up to 2% sporadic PD patients (Correia Guedes et al., 2010; Gilks et al., 2005; Tomiyama et al., 2008). Some of the missense mutations are also found in healthy control individuals (Carmine Belin et al., 2006; Kay et al., 2006; Lesage et al., 2008a). Gly2019Ser, the most frequent mutation in *LRRK2*, was reported worldwide with an average frequency of 1% in patients with sporadic PD and 4% in patients with hereditary PD (Chu and Kordower, 2007; Healy et al., 2008; Paisan-Ruiz et al., 2009). A heterogeneous distribution of this mutation in different ethnic groups was observed. The frequencies of this mutation is very low in Asian (<0.5%), higher in Caucasians (0.5–2.0% of sporadic PD and 5% of familial PD), and the highest (18–30%) in North African Arab and Ashkenazi Jewish populations (Benamer and de Silva, 2010; Chu and Kordower, 2007; Healy et al., 2008; Hulihan et al., 2008; Lesage et al., 2006; Ozelius et al., 2006; Zhang et al., 2009). The disease penetrance in carriers of *LRRK2* Gly2019Ser mutation is age-dependent. An international case-control consortium study conducted in 1045 people with *LRRK2* mutations from 133 families has reported the cumulative risk for carriers of Gly2019Ser mutation rises from 28% at age 59 years to 74% at age 79 years (Healy et al., 2008). Interestingly, the penetrance of Gly2019Ser in North African population is low and does not exceed 14% (Troiano et al., 2010). The worldwide and possible population-specific genetic penetrance of *LRRK2* mutations merit further exploration.

Multiple LRRK2 mutations (e.g. Arg1441Cys, Arg1441Gly, Tyr1699Cys, Gly2019Ser and Ile2020Thr) have been confirmed to be pathogenic. The substitutions of Gly2019Ser and Ile2020Thr increase the catalytic activity of the MAPKKK domain (Gloeckner et al., 2006; West et al., 2005) (Figure 1). In cellular models, overexpression of disease-causing LRRK2 mutants is toxic; the toxicity is associated with kinase activity, GTP-binding, and mitochondria-dependent apoptosis (Greggio et al., 2006; Iaccarino et al., 2007; Smith et al., 2006; West et al., 2005; West et al., 2007). The presence of mutations in healthy carriers and the high frequency in sporadic PD patients, together with the highly variable onset ages and disease phenotypes, render the determination of pathogenicity of the identified LRRK2 mutations extremely challenging. *post mortem* studies of PD patients with *LRRK2* mutations show pleomorphic pathology with typical Lewy bodies in most cases (Gilks et al., 2005; Ross et al., 2006; Zimprich et al., 2004).

2.4 Recessively inherited mutations

Loss-of-function mutations in genes encoding Parkin, PINK1, and DJ-1 cause early-onset autosomal recessive PD. All these forms of PD are relatively rare and are L-DOPA responsive. Simple mutations (e.g. missense, nonsense and splice site mutations), exonic rearrangements, small insertions and deletions, as well as copy number variations of the promoter region and exon(s) have been identified in these recessive genes. While

homozygous or compound heterozygous mutations in these recessive genes have a complete penetrance, the pathogenic effect of a single heterozygous gene defect in these genes is far less clear. The higher prevalence of the heterozygous variants in PD patients than in control individuals (Lesage et al., 2008b; Marongiu et al., 2008) implies that a heterozygous recessive mutation may predispose the carriers to PD. To date, no neuropathological studies have been reported for patients with *PINK1* and *DJ-1* mutations.

2.4.1 *Parkin* (*PARK2*) and *Parkin*—The transcript of *Parkin* spans approximately 1.38Mb of genomic DNA and comprises 12 exons. The 465 amino acid protein, *Parkin*, harbors an N-terminal ubiquitin-like domain, an IBR (in-between-ring) domain, and two RING-finger domains. *Parkin* functions as an E3-ligase, conjugating ubiquitin to excessive or dysfunctional proteins to target them for degradation by the proteasome (Chung et al., 2004; Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000) (Figure 1). *Parkin* modifies proteins through monoubiquitination and polyubiquitination using both lysine-48 and lysine-63 linkages (Dawson and Dawson, 2010). Under physiological conditions, *Parkin* also participates in mitochondrial maintenance (Berger et al., 2009; Exner et al., 2007; Park et al., 2006; Poole et al., 2008) and clearance of dysfunctional mitochondria by autophagy (Narendra et al., 2008) (Figure 1).

Loss-of function mutations in *Parkin* (>100 different mutations identified) are a major cause of early-onset autosomal recessive familial PD in different ethnic groups (Dawson and Dawson, 2010; Kitada et al., 1998). These mutations account for ~50% of early-onset familial PD cases and at least 20% of early-onset sporadic PD (Lucking et al., 2000). A complete penetrance appears in individuals who have two disease-causing mutations (homozygous or compound heterozygous) in *Parkin*. Individuals carrying a single heterozygous *Parkin* mutation present with minor Parkinsonian symptoms (Khan et al., 2005; Lesage et al., 2008b; Pramstaller et al., 2002), susceptibility to late-onset PD, as implied by the reduced uptake of ¹⁸F-labelled L-DOPA detected by PET scans (Klein et al., 2007; Pavese et al., 2009), or no neurological impairment in healthy control individuals (Bruggemann et al., 2009; Kay et al., 2007). Heterozygous promoter and coding polymorphisms in *Parkin* gene are associated with increased susceptibility to late-onset PD (Hu et al., 2000; Lucking et al., 2003).

Most PD-causing mutations in *Parkin* abolish its E3 ubiquitin ligase activity, while some missense mutations reduce this activity and affect substrate specificity (Dawson and Dawson, 2010; Sriram et al., 2005). Although numerous potential substrates for *Parkin* have been reported, it remains unclear which of these substrates mediate the pathogenicity of *Parkin* deficiency. Dopaminergic, oxidative, and nitrosative stress, key players in PD, can inactivate *Parkin* (Chung et al., 2004; Dawson and Dawson, 2010; LaVoie et al., 2005; Yao et al., 2004). About half of examined PD patients carrying causative *Parkin* mutations have Lewy bodies, while the other half do not (Dawson et al., 2010).

2.4.2 *PINK1* (*PARK6*) and *PINK1*—*PINK1* comprises 8 exons that code for 581 amino acids. *PINK1* contains an N-terminal mitochondrial targeting motif and a highly conserved serine/threonine kinase domain (Valente et al., 2004). *PINK1* is mainly localized to the mitochondria and its kinase domain appears to face the cytosol, which suggests that physiologic substrates of *PINK1* may reside in the cytosol (Silvestri et al., 2005; Zhou et al., 2008). However, the submitochondrial localization, the action site, and the authentic physiologic/pathophysiologic substrates of *PINK1* require further investigations. *PINK1* has been reported to participate in multiple mitochondrial functions, such as calcium dynamics, trafficking, respiration efficacy, free radical formation, and opening of the mitochondrial permeability transition pore (Gandhi et al., 2009; Liu et al., 2009; Plun-Favreau and Hardy, 2008; Weihofen et al., 2009; Yang et al., 2008). *PINK1* may regulate mitochondrial

response to cellular and oxidative stress, protecting cells from mitochondrial dysfunction (Pridgeon et al., 2007; Valente et al., 2004) (Figure 1).

Approximately 6.5% of known mutation carriers carry a homozygous recessive or compound heterozygous mutation in *PINK1* (Nuytemans et al., 2010). The *PINK1* mutation spectrum locates across the entire gene. Penetrance appears to be complete in individuals carrying two disease-causing mutations in *PINK1*, whereas the role of heterozygous mutations in *PINK1* is unsettled. Many putative pathogenic mutations were also observed in heterozygous state in familial and sporadic patients as well as in healthy control individuals (Bonifati, 2007; Djarmati et al., 2006; Healy et al., 2004; Klein et al., 2007). Patients with such mutations reveal a broad phenotypic spectrum ranging from early-onset parkinsonism with atypical features to clinical manifestations indistinguishable from sporadic late-onset PD (Farrer, 2006; Gelmetti et al., 2008). PET studies using ^{18}F -l-DOPA have demonstrated nigrostriatal abnormalities in heterozygous carriers and possibly early-onset disease (Khan et al., 2002; Valente et al., 2004). It is believed that loss-of-function mutations in *PINK1* influences its stability, localization, and kinase activity (Beilina et al., 2005; Petit et al., 2005; Valente et al., 2004).

2.4.3 DJ-1 (*PARK7*) and DJ-1—*DJ-1* contains 8 exons that span 24 kb. DJ-1, a member of the ThiJ/PfpI family of molecular chaperones, is expressed widely throughout the body (Cookson, 2010; Farrer, 2006; Moore et al., 2006). It primarily exists as a dimer and is subcellularly localized to the cytosol, mitochondrial matrix, and mitochondrial intermembrane space (Cookson, 2010; Tao and Tong, 2003; Zhang et al., 2005). DJ-1 is a redox-sensitive molecular chaperone with diverse functions (Kahle et al., 2009; Moore et al., 2006). It regulates redox-dependent kinase signaling pathways and antioxidant gene expression (Kahle et al., 2009) (Figure 1). Acting as an atypical peroxiredoxin-like peroxidase, DJ-1 protects mitochondria against oxidative stress (Andres-Mateos et al., 2007).

Recessively inherited *DJ-1* mutations are rare overall, causing less than 1% of early-onset parkinsonism (Clark et al., 2004; Lockhart et al., 2004). Both missense mutations and copy number variants of *DJ-1* have been identified in PD patients (Bonifati et al., 2003; Hague et al., 2003). Individuals carrying homozygous or compound heterozygous mutations in *DJ-1* present with reduced dimer formation or lack of expression of DJ-1 (Macedo et al., 2003; Moore et al., 2003). Clinically unaffected *DJ-1* mutation carriers who carry heterozygous variants seem normal in brain-imaging studies, indicating a requirement of a complete loss of DJ-1 function for PD (Dekker et al., 2004).

2.5 Crosstalk among PD-associated genetic pathways

Striking similarities in the phenotypes among familial PD patients carrying various PD-related gene mutants and sporadic PD patients strongly suggest shared molecular pathways and genetic interactions in the pathogenesis of both familial and sporadic PD (Figure 1). In support of this notion, *PINK1*, *Parkin* and *DJ-1* have been reported to functionally interact to maintain mitochondrial integrity and functionality and to protect against adverse effects of multiple stressors (Deng et al., 2008a; Exner et al., 2007; Narendra et al., 2010; Park et al., 2006; Poole et al., 2008; Vives-Bauza et al., 2010b) (Figure 1). Transgenic expression of *Parkin* attenuates *PINK1* loss-of-function phenotypes in flies, whereas transgenic expression of *PINK1* had no effect on *Parkin* loss-of-function phenotypes (Clark et al., 2006; Park et al., 2006). *PINK1* mediates the translocation of *Parkin* to mitochondria, which directs mitochondria to autophagy (Vives-Bauza et al., 2010a). Furthermore, *PINK1* also interacts with mitochondrial serine protease *HtrA2* (*PARK13*); *PINK1*-dependent phosphorylation of *HtrA2* might modulate the proteolytic activity of *HtrA2*, thereby contributing to an increased

resistance of cells to mitochondrial stress (Plun-Favreau et al., 2007). Coexpression of PINK1, Parkin or DJ-1, but not PD-associated mutants of these genes, rescues cells from mitochondrial fusion elicited by α -synuclein overexpression (Kamp et al., 2010). It has been proposed that the functional interaction among different PD-associated genes does not necessarily result from a direct physical interaction among corresponding proteins; alternatively, the convergence at the level of mitochondrial integrity of different pathways appears, at least partially, responsible for the overall outcome of such interactions. Moreover, DJ-1, Parkin, and PINK1 have been reported to form a ubiquitin E3 ligase complex promoting unfolded protein degradation (Xiong et al., 2009). In double transgenic *Drosophila*, the coexpression of PINK1, DJ-1 or Parkin alters various phenotypes of the eye and dopaminergic system elicited by LRRK2 expression in a complex fashion (Venderova et al., 2009). In α -synuclein/LRRK2 double mutant mice, overexpression of LRRK2 promoted and knockout of LRRK2 suppressed the abnormal aggregation and somatic accumulation of α -synuclein as well as the progressive loss of cortical and striatal neurons of the forebrain induced by overexpression of Ala53Thr α -synuclein (Lin et al., 2009). These findings reveal an important functional interplay between LRRK2 and α -synuclein and suggest these two dominant PD gene products may share common pathogenic mechanisms. Certainly, further investigation of detailed molecular cascades of genetic interactions will glean many fresh insights into PD pathogenesis (Figure 1).

2.6 Genome-wide association study (GWAS) of PD

Multiple GWASs conducted in PD patients and controls have independently identified polymorphisms in the *SNCA* (4q22), *MAPT* (17q21.1) and *LRRK2* (12q12) regions as risk factors for PD, confirming the known associations of these genes with PD (Edwards et al., 2010; Maraganore et al., 2005; Pankratz et al., 2009; Satake et al., 2009; Simon-Sanchez et al., 2009; Tan et al., 2010). A GWAS and two replication studies conducted in a total of 2,011 PD patients and 18,381 controls from Japan have detected two new risk loci: *1q32* (*PARK16*) and *4p15* (*BST1*) (Satake et al., 2009). While *SNCA* polymorphisms as a major risk locus across populations have been confirmed by several GWASs, *BST1* and *MAPT* as risk loci for PD show population differences (Satake et al., 2009; Simon-Sanchez et al., 2009). Thus, an unequivocal role for common genetic variants in the etiology of typical PD implies population-specific genetic heterogeneity in PD. Additionally, a recent GWAS of 2,000 PD patients and 1,986 controls from the NeuroGenetics Research Consortium Common has identified an association of genetic variation in the *HLA* region with late-onset sporadic PD (Hamza et al., 2010), which will be discussed in detail later (see Section 4.3). Certainly, GWASs in a population sample may prove to be productive in identifying association of genetic variability with disease risk. However, association does not imply causation and can not be used to predict disease risk of an individual for a certain disease. The pathogenic effects of specific mutations and polymorphisms must be investigated.

3. Genetically engineered PD animal models

Transgenic overexpression of mutant genes for autosomal-dominant genes (*SNCA* and *LRRK2*) and knockout/knockdown of autosomal-recessive genes (*Parkin*, *DJ-1*, and *PINK1*) in different organisms, such as mice, *Drosophila* and *C. elegans* have been used to model PD. These models not only yield fruitful insights into pathogenic mechanisms of causative gene mutations in neuronal dysfunction in familial PD, but also provide fresh insights into sporadic PD. Several excellent reviews have introduced in detail these animal models and discussed controversies around these models (Chesselet, 2008; Dawson et al., 2010; Lim and Ng, 2009; Lu and Vogel, 2009; Pienaar et al.). Here, we briefly summarize the genetically engineered animal models of PD, with focus on future work on refinement and optimization of these models.

3.1 Overexpression of α -synuclein

A variety of transgenic animals overexpressing either wildtype or mutant human α -synuclein variably recapitulate neurodegeneration and α -synuclein pathology (e.g. aggregation, truncation, phosphorylation and ubiquitination) in different brain regions (Farrer, 2006). For example, α -synuclein transgenic *Drosophila* (Feany and Bender, 2000) and virus-infected mice, rats, and primates (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002; Yamada et al., 2004) revealed loss of dopamine neurons and formation of Lewy body-like inclusions. However, of the many α -synuclein transgenic mouse models, a few showed alterations in the nigrostriatal dopamine system (Masliah et al., 2000; Richfield et al., 2002; Rockenstein et al., 2002; Tofaris et al., 2006), and only very few of them developed overt loss of dopamine neurons in the SN. For instance, when human wildtype or doubly mutated α -synuclein (A53T and A30P) was expressed in mice by using rat tyrosine hydroxylase promoter, the number of nigral dopamine neurons declines with age (Thiruchelvam et al., 2004). Although the reason for the lack of overt loss of nigral dopamine neurons in most human α -synuclein transgenic mice is unclear, we speculate the following reasons may be partially responsible for such a phenomenon. Firstly, while A53T mutant α -synuclein is pathogenic in humans, the threonine at position 53 is the wildtype amino acid of rodent α -synuclein; this difference and additional five amino acid differences between mouse and human α -synuclein may affect the oligomerization and fibrillogenesis as well as consequent pathogenicity of α -synuclein. Secondly, the coexistence of human α -synuclein and mouse α -synuclein is postulated to influence the phenotypes of human α -synuclein transgenic mice (Cabin *et al.*, 2005; Rochet *et al.*, 2000). Thirdly, abnormalities in genetically engineered mice carrying PD-related genetic defects (e.g. mutant *SNCA* and *LRRK2*), probably at the very best only mimic the earliest dysfunction of the nigrostriatal dopaminergic system and the initial preclinical stage of PD. Fourthly, the short lifespan of mice (< 3 years) makes it strictly difficult, if not impossible, to replicate the decades-long neurodegenerative process of PD patients in these animals. Fifthly, the presence of neuromelanin in dopamine neurons in humans and non-human primates, but not in rodents, may render primates more vulnerable than rodents to diverse PD-associated insults (e.g. genetic mutations and environmental toxins). Sixthly, one single genetic defect is not sufficient to produce overt loss of nigral dopamine neurons, and an environmental trigger may be required for PD neurodegeneration. These potential reasons except the first two may be also partially responsible for the paucity of the full-spectrum of PD in other genetic mouse models.

3.2 Overexpression of *LRRK2*

Drosophila and *C. elegans* overexpressing mutant *LRRK2* display death of dopamine neurons and increased sensitivity to rotenone and paraquat (Liu et al., 2008; Ng et al., 2009; Saha et al., 2009; Venderova et al., 2009). However, the short lifespan and the lack of endogenous α -synuclein expression render it potentially problematic to study mechanisms of *LRRK2*-mediated neurodegeneration in *Drosophila* and *C. elegans*, since a prominent feature of PD caused by *LRRK2* mutations is α -synuclein pathology. Bacterial artificial chromosome (BAC) transgenic mice overexpressing Arg1441Gly mutant *LRRK2* show diminished striatal dopamine, axonal pathology of nigrostriatal dopamine pathway, and age-dependent, levodopa-responsive motor impairment (Li et al., 2009). BAC transgenic mice overexpressing Gly2019Ser mutant *LRRK2* exhibited an age-dependent striatal dopamine deficiency but not degeneration of nigrostriatal dopamine neurons or terminals at 12 months of age (Li et al., 2010). Conditional expression of wildtype and Gly2019Ser mutant *LRRK2* in dopamine neurons or knockin of the Arg1441Cys mutant in mice failed to produce dopaminergic neurodegeneration (Lin et al., 2009; Tong et al., 2009). So far, the most consistent phenotypes in various *LRRK2* transgenic mice are abnormalities in the nigrostriatal dopamine neurotransmission. These abnormalities probably indicate early dysfunction of the nigrostriatal dopamine system. However, the lack of substantial

neurodegeneration in LRRK2 transgenic mice limits their utility in testing neuroprotective agents. Interestingly, co-overexpression of either wildtype or Gly2019Ser LRRK2 with A53T α -synuclein promoted abnormal somatic accumulation and aggregation formation of α -synuclein and dramatically accelerated neurodegeneration in cortical and striatal neurons of the forebrain in the double-transgenic mice (Lin *et al.*, 2009). However, the expression of the transgene in less than 5 percent of midbrain dopaminergic neurons and the occurrence of forebrain neurodegeneration in these double-transgenic mice limit their relevance to PD pathogenesis. Crossbreeding of inducible dopamine neuron-specific α -synuclein transgenic mice with LRRK2 mutant mice may provide an innovative and valuable mouse model to investigate how LRRK2 interacts with α -synuclein in the modulation of dopaminergic function and degeneration (Tong and Shen, 2009).

3.3 Knockout of α -synuclein or LRRK2

α -synuclein knockout mice exhibit a subtle electrophysiological phenotype but no neurodegenerative pathology (Perez and Hastings, 2004). Knockout of LRRK2 in mice is not neurotoxic *per se* and does not exacerbate MPTP-elicited dopaminergic neurodegeneration (Andres-Mateos *et al.*, 2009). Deletion of LRRK homolog in *Drosophila* causes dopaminergic impairment and locomotive deficit (Lee *et al.*, 2007). Knockdown of LRRK homolog in *C. elegans* does not affect on baseline viability but enhances their vulnerability to rotenone toxicity (Saha *et al.*, 2009).

3.4 Knockout of DJ-1

Drosophila possesses two orthologs of DJ-1: DJ-1a and DJ-1b. Knockout of both DJ-1 homologs in *Drosophila* does not alter fertility, lifespan, or dopamine neurons but sensitizes *Drosophila* to PD-associated environmental toxins, paraquat and rotenone (Meulener *et al.*, 2005). Selective knockdown of DJ-1 by RNAi in dopamine neurons leads to age-dependent loss of dopamine neurons in the dorsomedial cluster in *Drosophila* (Yang *et al.*, 2005). DJ-1 knockout mice exhibit abnormal dopamine neurotransmission in the nigrostriatal pathway and motor deficits but no observable neurodegeneration (Andres-Mateos *et al.*, 2007; Chen *et al.*, 2005b; Goldberg *et al.*, 2005; Kim *et al.*, 2005).

3.5 Knockout of Parkin or overexpression of mutant Parkin

Parkin-knockout *Drosophila* reveals male sterility, reduced lifespan, and severe defects in flight and climbing abilities. These phenotypes may be associated with mitochondrial defects and consequent apoptotic cell death in muscle and sperm. Aged Parkin-null flies show dopamine-responsive locomotion deficits and degeneration of a subset of dopamine neurons (Greene *et al.*, 2003; Whitworth *et al.*, 2005). Parkin-null mice display either subtle alterations in the nigrostriatal dopamine circuit or no overt dopaminergic or behavioral abnormalities (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Perez and Palmiter, 2005). Interestingly, overexpression of mutant human Parkin in *Drosophila* and mice causes progressive degeneration of dopamine neurons (Lu *et al.*, 2009; Sang *et al.*, 2007; Wang *et al.*, 2007).

3.6 Knockout of PINK1

PINK1-null *Drosophila* and Parkin-null *Drosophila* share marked phenotypic similarities including male sterility, behavioral deficits, fewer dopamine neurons, mitochondrial defects, and apoptosis in muscle (Clark *et al.*, 2006; Gautier *et al.*, 2008; Park *et al.*, 2006). In the *Drosophila* loss-of-function models of PINK1 and Parkin, the dopaminergic system is mildly affected, while the most dramatic phenotypes occur outside the nervous system. PINK1 knockout mice show subtle deficits in nigrostriatal dopamine neurotransmission (Gautier *et al.*, 2008; Gispert *et al.*, 2009; Kitada *et al.*, 2007). Because deficiency in Parkin,

PINK1, or DJ-1 in mice does not cause substantial nigrostriatal pathology, these mice at the very best only mimic the earliest dysfunction of the nigrostriatal dopaminergic system. Thus, usage of current autosomal-recessive animal models to study mechanisms of dopamine neurodegeneration and to screen neuroprotective agents is potentially problematic or impossible. Overall, gene-based *Drosophila* and *C. elegans* models offer powerful tools to rapidly screen genetic and pharmacologic modifiers of known PD-associated genes, but the challenge is to verify the evolutionary conservation of these potential modifiers in human PD.

4. The environmental causes of PD

4.1 Pesticides and other environmental toxins

Although modern genetics uncovers important genetics basis in familial PD, it is worth emphasizing that only about 10%–20% of PD cases result from genetic causes. The majority of PD cases are sporadic and idiopathic. The accidental use of 1-methyl-4-phenyl-1,2,3-tetrahydropyridine (MPTP), a by-product of illicit heroin synthesis, leads to a Parkinson syndrome that is clinically indistinguishable from PD (Langston *et al.*, 1983). This milestone finding has stimulated the search for environmental factors as potential causes of PD. The structural similarity between paraquat (1,1'-dimethyl-4,4'-bipyridinium), a common herbicide, and 1-methyl-4-phenylpyridinium ion (MPP⁺), the active metabolite of MPTP, prompted speculation that paraquat might be a dopaminergic neurotoxicant and exposure to paraquat may be related to the development of PD. Epidemiological studies have reported that exposure to pesticides including paraquat correlates with increased incidence of PD (Ascherio *et al.*, 2006; Liou *et al.*, 1997; Rajput and Uitti, 1987). Case reports have also associated development of Parkinsonism with exposure to maneb (a widely used fungicide) and organophosphorus insecticides (Bhatt *et al.*, 1999; Liu *et al.*, 2003; Meco *et al.*, 1994). Elevated levels of residual dieldrin (an organochlorine pesticide) and polychlorinated biphenyls (widely used industrial chemicals) have been detected in the brains of PD patients (Corrigan *et al.*, 1998; Corrigan *et al.*, 2000; Fleming *et al.*, 1994). Chronic occupational exposure to high levels of manganese in manganese miners causes accumulation of this metal in the basal ganglia, resulting in tremors, rigidity and psychosis that resemble PD (Mergler and Baldwin, 1997). However, elevated levels of certain pesticides or chemicals in the brains of PD patients do not indicate causal relationship between toxin exposure and PD development. Experimental exposure of animal models to several classes of pesticides, such as paraquat, dithiocarbamate-based fungicides (e.g. maneb), insecticide rotenone, and organochlorine pesticides (e.g. dieldrin) leads to dopaminergic neurotoxicity. These pesticides have hence been proposed as potential PD risk factors in humans (Liu *et al.*, 2003). Furthermore, epidemiological and case-control studies have suggested rural residence, well-water consumption, pesticide use, and certain occupations (e.g. farming, mining and welding) are associated with an increased risk of PD (Dhillon *et al.*, 2008; Elbaz *et al.*, 2009; Engel *et al.*, 2001; Gorell *et al.*, 1998; Semchuk *et al.*, 1992). Several specific pesticides including dieldrin, maneb, paraquat, and rotenone have recently been implied to increase PD risk by recent epidemiological studies (Brown *et al.*, 2006; Dhillon *et al.*, 2008; Kamel *et al.*, 2007; Ritz *et al.*, 2009). However, overall epidemiological evidence is fragmentary and insufficient to establish a specific association between exposure to specific pesticides/toxicants and increased risk for PD.

4.2 Brain trauma

Numerous case reports have associated episodes of traumatic brain injuries with the occurrence of an acute-phase Parkinsonism or later-life development of PD (Doder *et al.*, 1999; Nayernouri, 1985). Several epidemiological studies including an analysis of the medical histories of a large group of World War II veterans have identified antecedent head

injuries as a major risk factor for the development of PD in later life (Goldman *et al.*, 2006; Stern, 1991; Taylor *et al.*, 1999). However, contradiction epidemiological results suggest no such a correlation (Goetz and Pappert, 1992; Spangenberg *et al.*, 2009; Williams *et al.*, 1991). Boxers who have professionally related higher chance of suffering head injuries appear to have a higher incidence of Parkinson-like movement disorders (Friedman, 1989; Guterman and Smith, 1987).

4.3 Inflammation

Early-life occurrence of brain inflammation resulting from either brain injury or exposure to infectious agents may modulate PD risk (Figure 1). Epidemiologic studies pointed to an association between early-life viral infections and postencephalitic PD, while case reports tend to relate viral infections to the development of acute Parkinsonism (Duvoisin *et al.*, 1972; Elizan and Casals, 1991; Ghaemi *et al.*, 2000; Henry *et al.*, 2010; Liu *et al.*, 2003). Experimental exposure of neonatal Fisher 344 rats to Japanese encephalitis virus can induce degeneration of nigral dopamine neurons, gliosis in the SN, and movement disorders resembling human PD (Ogata *et al.*, 1997). Moreover, prospective studies suggest that higher plasma concentration of proinflammatory cytokine interleukin-6 correlates with an increased risk for PD (Chen *et al.*, 2008). Epidemiological and case-control studies on associations between chronic use of nonsteroidal anti-inflammatory drugs and PD risk generate mixed results, with some studies suggesting a reduced incidence and others finding no association (Chen *et al.*, 2005a; Chen *et al.*, 2003; Powers *et al.*, 2008; Ton *et al.*, 2006). DNA polymorphisms of several inflammatory cytokines might become risk factors for PD (Wahner *et al.*, 2007). A recent GWAS of 2,000 PD patients and 1,986 controls from the NeuroGenetics Research Consortium Common identifies an association of genetic variation in the *HLA* region with late-onset sporadic PD (Hamza *et al.*, 2010). This hypothesis-free GWAS lends strong and independent support to the participation of inflammation in PD pathogenesis (Figure 1). *HLA* (human leukocyte antigen) region contains a large number of genes related to immune function in humans. HLA class II histocompatibility antigen plays a central role in inflammation by presenting peptide antigens to the immune system. While additional studies are needed to establish a causal role for inflammation in PD pathogenesis, the increased awareness of the involvement of inflammation in PD will stimulate research toward new therapeutic targets. Collectively, environmental risk factors, including toxins, early-life infections in the brain, and traumatic brain injuries may contribute to the cause of PD (Figure 1). However, specific causative environmental toxicants or infectious agents (DNA or antigens) for PD have not yet been identified.

4.4 Protective environmental factors

Inverse associations of PD with cigarette smoking, caffeine intake, and high plasma urate concentrations have been noted (Checkoway *et al.*, 2002; Chen *et al.*, 2010; Hernan *et al.*, 2002; Powers *et al.*, 2008; Weisskopf *et al.*, 2007). However, whether such associations reflect true biologic protection needs further investigation. Interestingly, a few randomized controlled clinical trials suggest beneficial effects of exercise (e.g. improvements in postural stability and balance task performance) in PD patients (Dibble *et al.*, 2009; Gobbi *et al.*, 2009; Morris *et al.*, 2009). Larger number of studies, more outcomes used, and longer term follow-up are needed to confirm that exercise-related improvements in PD motor dysfunction are long-lasting. A few prospective cohort studies suggest that moderate to vigorous exercise may protect against PD (Xu *et al.*, 2010). In rodents, exercise reduces the behavioral impairments and the loss of dopamine neurons elicited by the dopaminergic neurotoxins MPTP or 6-hydroxydopamine (6-OHDA) (Gerecke *et al.*, 2010; Zigmond *et al.*, 2009). The trigger of endogenous neuroprotective mechanisms (e.g. increased expression of neurotrophic factors) and the preconditioning (mild stress before more intensive stress) have been proposed to be responsible for beneficial effects of exercise (Zigmond *et al.*, 2009).

5. Toxin-based models of PD

5.1 MPTP model

Several toxin-based models of PD have provided the framework for symptomatic improvements in medical and surgical therapies for PD. MPTP model is the most widely used PD model for the study of molecular cascades of death of dopamine neurons and for the screening of neuroprotective strategies. MPTP can cross the blood-brain barrier after subcutaneous or intraperitoneal injection. Once inside the brain, MPTP is converted into MPP⁺ by the enzyme monoamine oxidase B in astroglia. Released MPP⁺ is then taken up by the dopamine transporter and concentrated in dopamine neurons where it inhibits mitochondrial complex I, resulting in ATP depletion, increased free-radical generation, and death of dopamine neurons. MPTP is the only known dopaminergic neurotoxin inducing Parkinsonism in both humans and monkeys (Jackson-Lewis and Przedborski, 2007). Mice and monkeys treated with MPTP develop reliable and reproducible nigrostriatal lesion and gliosis (Jackson-Lewis and Przedborski, 2007). MPTP-injected monkeys revealed increased brain levels of α -synuclein, PD-like modifications of α -synuclein (e.g. aggregation, nitration and phosphorylation), or intraneuronal inclusions (Forno et al., 1986; McCormack et al., 2008; Purisai et al., 2005). However, it seems that unless MPTP is chronically infused by osmotic minipump or is administered with probenecid, a uricosuric agent that is used to block the rapid clearance of MPTP, proteinaceous inclusions will not be produced in dopamine neurons in mice (Fornai et al., 2005; Meredith et al., 2002; Shimoji et al., 2005). A few drawbacks, such as strain-specificity of animals, lack of classical and convincing α -synuclein pathology, relatively rapid kinetics of neurodegeneration and partial lesion recovery, make MPTP mouse models less ideal for studying PD progression and for screening drugs to slow down the disease progression.

5.2 6-OHDA model

6-OHDA is the first catecholaminergic neurotoxin that was used to generate animal models of PD. Because of its preferential uptake by dopamine and noradrenergic transporters, 6-OHDA induced relatively selective neurotoxicity to monoaminergic neurons (Ungerstedt, 1968). Inside these neurons, 6-OHDA produces reactive oxygen species and quinones that inactivate biological macromolecules. Being unable to cross the blood-brain barrier, 6-OHDA must be administered by local stereotaxic injection to target the nigrostriatal dopaminergic pathway (Javoy et al., 1976). When injected into the SN or median forebrain bundle that carries ascending dopaminergic and serotonergic projections to the forebrain, 6-OHDA causes acute loss of dopamine neurons within 24 hr (Jeon et al., 1995); when injected into the striatum, it induces retrograde degeneration of nigral dopamine neurons, which lasts for 1–3 weeks (Przedborski et al., 1995; Sauer and Oertel, 1994). To date, no Lewy body-like inclusion has been described in 6-OHDA model. The paucity of classical PD pathology, the relatively acute neurotoxicity, and the requirement of stereotaxic injection make 6-OHDA model less popular in recent years.

5.3 Rotenone model

Rotenone, a mitochondrial complex I inhibitor (Earley and Ragan, 1984), is commonly used as a pesticide and fish poison. Although it remains to be determined whether typical human exposure to rotenone alone is likely to increase risk for PD, rotenone has been employed to model PD. Intracerebral application of rotenone led to damage to the nigrostriatal dopaminergic pathway in rats (Heikkila et al., 1985). Initial attempts to systemically administer rotenone, acutely or sub-chronically, to rodents produced only minimal damage to the nigrostriatal dopaminergic system (Ferrante et al., 1997; Thiffault et al., 2000). Later, continuous systemic administration of rotenone to rats and mice reproduces cardinal features of PD, including the degeneration of the nigrostriatal dopaminergic pathway, the formation

of Lewy body-like inclusions containing ubiquitin and filamentous α -synuclein in remaining nigral neurons, and movement impairments (Alam and Schmidt, 2002; Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003). Mechanistically, inhibition of mitochondrial complex I activity with consequent ATP depletion and oxidative damage, facilitation of α -synuclein aggregation and inflammation-induced injury may be responsible for the dopaminergic neurotoxicity of rotenone (Betarbet et al., 2000; Gao et al., 2002a; Sherer et al., 2002; Sherer et al., 2003). A high mortality has limited an extensive utility of rotenone model.

5.4 Paraquat model

Intracerebral injection of paraquat resulted in loss of nigral dopamine neurons, depletion of dopamine in the striatum, and an elevated response to apomorphine-induced rotational behavior (Liou et al., 1996). Effects of systemic administration of paraquat on dopamine neurons have been less consistent. Paraquat does not easily penetrate the blood brain barrier (Shimizu et al., 2001), and its distribution in the CNS is not region-specific or cell-type-specific. Mice that underwent a single or repeated systemic administration of paraquat exhibited a selective loss of nigral dopamine neurons with or without degeneration of striatal dopamine fibers or a reduction in striatal dopamine content (Brooks et al., 1999; Manning-Bog et al., 2002). Free radical generation and redox cycling (Bonneh-Barkay et al., 2005; Fukushima et al., 1995; Wu et al., 2005; Yumino et al., 2002), α -synuclein upregulation and fibrillation (Manning-Bog et al., 2002; Uversky et al., 2001), as well as apoptotic cell death (Chun et al., 2001) have been associated with paraquat-induced dopaminergic neurotoxicity. Furthermore, repeated systemic administration of maneb and paraquat to mice induced synergistic nigrostriatal dopaminergic neurodegeneration and motor behavioral abnormalities (Thiruchelvam et al., 2000). Neonatal exposure to both maneb and paraquat sensitized the nigrostriatal dopaminergic system to rechallenge with the same agents during adulthood (Thiruchelvam et al., 2002).

5.5 Inflammation-associated model

Whether bacterial or viral infection can act as an initiating factor in human PD is unclear, but intracranial or systemic administration of bacterial endotoxin or virus into rodents has been used to produce inflammation-associated PD model. Direct nigral injection of lipopolysaccharide (LPS), the active immunostimulant in the cell wall of Gram-negative bacteria, causes robust microglial activation and acute loss of nigral dopamine neurons in rodents (Castano et al., 1998; Kim et al., 2000; Liu et al., 2000; Tomas-Camardiel et al., 2004). Chronic infusion of LPS for 2 weeks into the rat SN triggered a rapid activation of microglia, followed by a selective and gradual loss of nigral dopamine neurons that began at between 4 and 6 weeks and reached 70% by 10 weeks (Gao et al., 2002b). This is the first report that microglial activation induced by chronic exposure to inflammogen was capable of producing a delayed, progressive and selective degeneration of nigral dopamine neurons. Microglia-originated free radicals play a critical role in dopaminergic neurotoxicity in this inflammation-mediated model of PD (Gao et al., 2002b). Additionally, in utero exposure of developing rat fetuses to LPS results in lesions of nigrostriatal dopaminergic system in neonates (Ling et al., 2002). Adult rats with prenatal exposure to LPS developed progressive dopaminergic neurodegeneration following chronic nigral LPS infusion (Ling et al., 2006). Moreover, an intraperitoneal injection of LPS (5 mg/kg) led to significant loss of nigral dopamine neurons in C57BL/6 mice 7 months after the injection; such neurodegeneration progressed further overtime (Qin et al., 2007). These findings together indicate that once reaching certain threshold, the inflammatory process is capable of initiating neurodegeneration. The higher density of microglia in the SN than other brain regions (Kim et al., 2000; Lawson et al., 1990) may be partially responsible for the foremost loss of dopamine neurons in the inflammation-induced neurodegenerative process.

Although humans infected by highly pathogenic H5N1 influenza virus show acute neurological impairments ranging from mild encephalitis to motor disturbances to coma (de Jong et al., 2005; Gambotto et al., 2008), the long-term neurological manifestations of H5N1 infection among surviving hosts remain unknown. A recent study has reported that after intranasal inoculation in C57BL/6 mice, H5N1 virus travelled from the peripheral nervous system into the CNS. Microglial activation and α -synuclein phosphorylation and aggregation persists long after resolution of the infection in infected brain regions. While H5N1 virus was no longer detectable in the CNS 21 days post-inoculation, a significant loss of nigral dopamine neurons was observed 60 days after the virus infection (Jang et al., 2009). This study provides important information on the role of virus-associated inflammation in potential long-term impairment to the CNS. However, the requirement of a biosafety level 3⁺ laboratory will limit the usage of this virus-based PD model.

6. Two-hit PD models created by genetic lesions and environmental insults

As described above, animal models of PD created by using individual neurotoxins or genetic manipulation have been extremely valuable for studying the pathogenesis of PD. However, most of the current genetic PD models failed to reproduce overt nigrostriatal dopaminergic loss, suggesting that a single genetic risk factor is not sufficient and an environmental trigger may be required for producing nigral dopamine neuronal loss. Thus, two-hit animal models are generated to test whether genetic deficiency increases vulnerability of dopamine neurons to potential environmental risk factors (Horowitz and Greenamyre, 2010). The concept of “model fusion” in the creation of a new generation of animal models to study gene-environment interactions in PD has been proposed by Drs. Manning-Bog and Langston (Manning-Bog and Langston, 2007). These new models would be of tremendous importance to a more in-depth understanding of the pathogenesis of PD and to the discovery of therapeutics that can retard PD progression.

6.1 LPS and α -synuclein

The fact that only very few of many different α -synuclein transgenic mouse models developed loss of dopamine neurons in the SN reveals the limitations of rodent models in the replication of all aspects of age-related neurodegeneration. Alternatively, such a fact might also suggest that other factors participate in the development of PD. Stereotaxic injection of LPS into the SN of wildtype mice and transgenic mice overexpressing either wildtype or A53T mutant human α -synuclein induced similar nigral inflammatory reactions. Transgenic expression of human α -synuclein renders dopamine neurons more vulnerable to LPS-induced inflammation, which in turn leads to accumulation of insoluble α -synuclein aggregates in nigral neurons and death of nigral dopamine neurons. Nitrated/oxidized α -synuclein was detected in Lewy body-like cytoplasmic inclusions in LPS-injected transgenic mice (Gao et al., 2008). This two-hit (neuroinflammation and overexpression of wildtype or mutant human α -synuclein) animal model not only provides better tool to advance our understanding of the role of neuroinflammation and α -synuclein dysfunction in the pathogenesis of PD, but also underscores synergistic effects of genetic predisposition and environmental exposures in the development of PD. Furthermore, a two-hit, chronic, progressive model has been created by an intraperitoneal injection of a relatively low dose of LPS (3×10^6 EU/kg) into 7-month-old transgenic mice overexpressing A53T mutant human α -synuclein (Gao et al., 2011). Such a LPS injection induced similar systemic inflammation and a comparatively mild neuroinflammatory response in both wildtype and α -synuclein transgenic mice. However, only in LPS-injected transgenic mice, the low-grade neuroinflammation sustains; α -synuclein accumulates and forms aggregates; Lewy body-like neuronal inclusions containing aggregated α -synuclein occur; and dopaminergic neurodegeneration in the nigrostriatal pathway progresses over time. Thus, synergistic effects of low-grade neuroinflammation and α -synuclein dysfunction drive chronic PD

neurodegeneration, while neither factor alone is sufficient to cause neuronal death (Gao *et al.*, 2011). This new model mimics PD multifactorial etiology and recapitulates the key features of PD. More importantly, the chronic progressive nature of dopaminergic neurodegeneration makes this model invaluable for the study of mechanisms of PD progression and for the screening of disease-modifying therapeutics.

6.2 LPS and Parkin

Although loss-of-function mutations in the *parkin* gene cause early-onset familial PD, Parkin-deficient mice do not display nigrostriatal degeneration. The intraperitoneal administration of low-dose of LPS (7.5×10^5 EU/kg) twice a week into Parkin-null or wildtype mice for 3 to 6 months indicated that genetic deficiency in Parkin sensitizes nigral dopamine neurons to inflammation-mediated neurotoxicity. Specifically, although persistent neuroinflammation and oxidative stress responses triggered by LPS are not different between two genotypes, only Parkin-null mice displayed selective loss of nigral dopamine neurons and subtle fine-motor deficits (Frank-Cannon *et al.*, 2008). This model may enable identification of early biomarkers of neurodegeneration and preclinical screening of potential compounds that can delay the progressive degeneration of the nigrostriatal pathway.

6.3 Neurotoxins and α -synuclein

The possible interaction between genetic factors and neurotoxins has been investigated by examining the sensitivity of α -synuclein transgenic mice to secondary neurotoxic insults, such as paraquat, maneb, MPTP, or rotenone. In general, either dopaminergic neurodegeneration or α -synuclein pathology is missing or has not been determined in those neurotoxin-exposed α -synuclein transgenic mice. For instance, intraperitoneal injection twice a week for 3 weeks into transgenic mice overexpressing A53T mutant human α -synuclein with maneb and paraquat, but not with either pesticide alone, drastically exacerbates neuronal α -synuclein pathology (e.g. accumulation of aggregated α -synuclein and formation of α -synuclein-containing inclusions) throughout the CNS. However, no noticeable neuron loss in the SN or other brain regions was observed in transgenic mice after treatment with maneb and/or paraquat (Norris *et al.*, 2007). Whether the exacerbated α -synuclein pathology in pesticide-exposed transgenic mice represents early disease stage that precedes neuronal degeneration remains to be determined. In addition, weekly intraperitoneal injections of paraquat (10 mg/kg, 3 times) into wildtype and transgenic mice expressing human wildtype or A53T mutant human α -synuclein driven by the tyrosine hydroxylase promoter resulted in an upregulation of α -synuclein and the formation of α -synuclein-positive inclusions. However, only wildtype mice, but not transgenic mice, developed nigral degeneration after paraquat injection (Manning-Bog *et al.*, 2003). The mechanism underlying the protective effect of transgenic overexpression of human α -synuclein against pesticide-induced neurodegeneration in transgenic mice warrants further investigation. Interestingly, recent evidence also indicates that transgenic expression of α -synuclein abolishes the lethality and neurodegeneration induced by ablation of cysteine-string protein- α (CSP α , an abundant synaptic vesicle protein with a cochaperone function), whereas deletion of endogenous synucleins exacerbates these phenotypes. Here, transgenic α -synuclein attenuates CSP α -deficiency-elicited inhibition of SNARE-complex assembly, in which binding of α -synuclein to synaptic vesicle phospholipids is required (Chandra *et al.*, 2005).

Chronic treatment with MPTP (daily intraperitoneal injection of 16 or 30 mg/kg MPTP for 5 consecutive days), but not with rotenone (0.125 mg/kg twice a week for 3 consecutive weeks), led to greater deterioration of the nigrostriatal dopamine system in transgenic mice expressing human A30P mutant α -synuclein than in wildtype littermates (Nieto *et al.*, 2006).

Transgenic mice expressing wildtype or a doubly mutated (A53T and A30P) α -synuclein show increased density of the dopamine transporter and elevated vulnerability to MPTP neurotoxicity. The doubly mutated mice exhibit reduced locomotor responses to repeated doses of amphetamine (Richfield et al., 2002). Collectively, although neurotoxin-exposed α -synuclein transgenic mice reveal either exacerbated α -synuclein pathology or greater deterioration of nigrostriatal dopamine pathway, it seems there is dissociation between toxicant-induced α -synuclein pathology and neurodegeneration

6.4 Neurotoxins and LRRK2

The ubiquitous expression of LRRK2 increases lifespan and fertility of the *Drosophila* but enhances the sensitivity of these flies to rotenone (Venderova et al., 2009). Similar to α -synuclein knockout mice, LRRK2 knockout mice that lack the kinase domain of LRRK2 have no observable neurodegenerative pathology. Different from α -synuclein knockout mice that are more resistant to neurotoxicity induced by LPS or MPTP (Dauer et al., 2002; Gao et al., 2008), LRRK2 knockout mice display the same susceptibility to MPTP as wildtype mice (Andres-Mateos et al., 2009).

6.5 Glial cell line-derived neurotrophic factor (GDNF) and methamphetamine

The importance of GDNF for maintenance of dopamine neurons has been well established. Enhanced dopaminergic cell survival and fiber outgrowth as well as improved behavioral recovery in rodent and monkey models of PD have encouraged the use of GDNF in clinical trials. Safety concerns for CNS delivery of GDNF and mixed results from limited human studies make it impossible to draw a firm conclusion about the efficacy and potential usage of GDNF in PD treatment. The genetic reduction of *Gdnf* and the reduced availability of GDNF protein in young *Gdnf*^{+/-} mice increase the vulnerability of dopamine systems to methamphetamine exposure (Boger et al., 2010). This dual-hit model will extend current knowledge of the impact of GDNF on progressive dopaminergic degeneration and motor dysfunction in PD. Experimental exposure of these *Gdnf*^{+/-} mice to more relevant environmental toxins (e.g. rotenone or paraquat) may provide fruitful information on PD etiology and pathogenesis.

7. Ideal models of PD

To date, none of existing animal models of PD mirrors the full-spectrum of the disease, even though they reproduce many of the features of PD (Chesselet et al., 2008). What are ideal animal models of PD? Despite the paucity of Lewy bodies in some PD patients carrying mutant *LRRK2* or *Parkin*, all PD patients ultimately develop gradual loss of dopamine neurons in the nigrostriatal pathway. Therefore, any “reasonable” model of PD should replicate the loss of nigral dopamine neurons. Ideal PD models should be age-dependent and progressive. They should reproduce the gradual loss of dopamine neurons and the dopamine-responsive motor impairment (e.g. slowness of movement, rigidity, rest tremor and postural instability). These models should also exhibit Lewy bodies and Lewy neurites that contain aggregated α -synuclein and ubiquitin-proteasomal proteins. Chronic neuroinflammation, a common feature shared by neurodegenerative diseases, should be another key feature of ideal PD models. More importantly, ideal models should mimic the multifactorial etiology of PD, in which sub-toxic concentrations of relevant environmental toxins are administered into genetically engineered animals with PD-associated genetic defects. In addition, the pathological alterations in the autonomic and/or enteric nervous systems in PD patients are also desirable characteristics of ideal models. Collectively, ideal animal models of PD should recapitulate the dopamine-responsive motor impairment, the autonomic dysfunction, and the full pathological spectrum seen in PD patients.

8. Gene-environment interactions influence PD susceptibility

The concept that gene-environment interactions affect PD susceptibility has been proposed for more than a decade (Ross and Smith, 2007; Vance *et al.*, 2010). Only a few human association studies have examined gene-environment interactions, although many studies have described positive associations between genetic polymorphisms and increased risk for PD. To date, dozens of single-nucleotide polymorphisms (SNPs) in several genes have shown gene-environment interactions in relation to PD (Table 1). Cumulative and interactive effects of cigarette smoking and gene polymorphisms in *NOS1* (neuronal nitric oxide synthase), *NOS2A* (inducible nitric oxide synthase) or *SNCA* may modulate risk for PD (Hancock *et al.*, 2006; Hancock *et al.*, 2008; Levecque *et al.*, 2003; McCulloch *et al.*, 2008). Occupational pesticide exposure as well as high exposure to paraquat and maneb in carriers of *DAT* (dopamine transporter) genetic variants increased PD risk (Kelada *et al.*, 2006; Ritz *et al.*, 2009). SNPs in *NOS1* and *GSTP1* (glutathione S-transferase pi 1) have been linked to an increased risk for PD in pesticide-exposed individuals (Hancock *et al.*, 2008; Menegon *et al.*, 1998). However, the association between *GSTP1* and pesticide exposure has not been supported by a large cohort study conducted later (Dick *et al.*, 2007). Additional interactions between *GSTM1* (glutathione S-transferase Mu 1) null genotype and heavy solvent exposure (Dick *et al.*, 2007) and between *APOE* (apolipoprotein E) SNPs and coffee consumption (McCulloch *et al.*, 2008) have been found to affect PD susceptibility. However, the majority of gene-environment analyses in a case-control study of 767 PD patients and 1989 controls across five European centers did not show significant interactions between polymorphisms in 15 genes that influence metabolism of foreign chemicals or dopamine and exposure to solvents, pesticides and metals (Dick *et al.*, 2007). Overall, results from human association studies should be interpreted with caution as most of these studies are based on a relatively small number of exposed subjects. Certainly, more large-scale human association studies aimed at identifying gene-environment interactions in the development PD may prove to be fruitful.

9. Oxidative stress serves as a central mechanism of chronic PD progression

Aging represents an unequivocal risk factor for PD, and oxidative stress is believed to be a major component of age-related disorders such as cancer, strokes, osteoarthritis, cardiovascular and neurodegenerative diseases. Oxidative stress engages in diverse cellular processes and plays a prominent role in the induction of neuronal death. For instance, excessive free radicals can damage proteins (e.g. abnormal modification of α -synuclein and inactivation of Parkin), lipids, DNA, or RNA, leading to cell dysfunction (e.g. ubiquitin-proteasome system [UPS] and mitochondrial impairment) and eventual cell death. While overwhelming evidence underlines the importance of oxidative stress in dopaminergic neurodegeneration elicited by inflammation or various environmental toxins (e.g. MPTP, rotenone, paraquat and 6-OHDA), growing experimental findings indicate that all known PD-associated genetic defects (i.e., mutations in *SNCA*, *Parkin*, *PINK1*, *DJ-1*, and *LRRK2*) are related to oxidative stress directly or indirectly, which is discussed in each corresponding section above.

As illustrated in Figure 1, oxidative stress interacts with every aspect of major PD pathological events including protein misfolding and aggregation, mitochondrial dysfunction and impairment, UPS perturbation, and aberrant signal transduction. Given their intimate relationship, oxidative stress and mitochondrial impairment/dysfunction become integral and inseparable elements of PD pathogenesis. Emerging evidence has also demonstrated a detrimental role of oxidative stress in the disruption of intracellular protein homeostasis. For instance, free radicals can either cause oxidation of α -synuclein and parkin, thereby

exacerbating their misfolding and accumulation, or attack protein surveillance machineries by oxidizing their subunits or key enzymes (e.g. ubiquitin-activating [E1], -conjugating [E2], and -ligating [E3] enzymes, three enzymes responsible for protein ubiquitination). When the accumulation of misfolded proteins exceeds the capacity for the chaperone, ubiquitin–proteasome, and autophagy systems to detect, to repair, and eventually to destroy those faulty proteins (Box 1), especially when these protein surveillance machineries are malfunction under oxidative condition, the build-up of toxic misfolded proteins in the cell will precipitate cellular demise. Although the precise mechanism of UPS dysfunction in PD merits further investigation, this ATP-driven degradation machinery is sensitive to age-related energy depletion and oxidative stress (Tan *et al.*, 2009) as well as environmental toxins, particularly those that inhibit mitochondrial function or generate reactive free radicals (Betarbet *et al.*, 2006; Wang *et al.*, 2006). Oxidative stress can also affect the function of other key PD-linked gene products (e.g. DJ-1, PINK1 and LRRK2) and other signaling molecules, leading to abnormal signal transduction. Thus, oxidative stress appears as a converging point of multiple pathways underlying the PD pathogenesis and plays a central role in the chronic progression of PD.

Text box 1

protein surveillance machineries

Under physiological condition, the chaperone, ubiquitin–proteasome, and autophagy systems function in concert to effectively counterbalance the threat of protein misfolding and aggregation and to maintain protein homeostasis. Among these three major protein surveillance machineries, the chaperone system (i.e., molecular chaperones that include members of the heat-shock protein family) plays a major role in correctly folding and refolding proteins (Liberek *et al.*, 2008), sensing misfolded proteins, and directing misfolded proteins for proteolysis when a native folding state can not be achieved. In this case, misfolded proteins are usually tagged with ubiquitin(s) and degraded by the proteasome, the major intracellular protein degradation machinery that destroys unneeded or damaged proteins by proteolysis. A polyubiquitin chain on a substrate typically acts as a signal for degradation by the proteasome. Polyubiquitin modification of proteins regulates many essential cellular processes including protein degradation, membrane trafficking, cell cycle, gene transcription, DNA repair, and responses to oxidative stress. Impaired USP and disrupted ubiquitin signaling may cause broad consequences for neuronal function and survival. Under conditions of cellular stress, especially when the UPS is impaired, autophagy serves as a compensatory degradation system; polyubiquitinated proteins can also be removed via the autophagy–lysosomal pathway (Pandey *et al.*, 2007). This autophagy–lysosomal pathway appears as a final line of defense against the accumulation of toxic misfolded proteins in the cell, and it participates in the maintenance of a balance between the synthesis, degradation, and subsequent recycling of cellular products. This protein degradation process involves the sequestration of damaged organelles and unwanted protein substrates into a double-membrane vesicle called autophagosome. The autophagosome then fuses with a lysosome to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases. This form of autophagy is called macroautophagy. Another form of autophagy is chaperone-mediated autophagy, which involves the direct translocation of substrate/chaperone complex—composed of unfolded proteins and a cytosolic chaperone hsc70—across the lysosomal membrane (Martinez-Vicente *et al.*, 2008; Mizushima *et al.*, 2008).

10. Conclusion

Emerging evidence has demonstrated an intimate connection between genetic and environmental causes of PD neurodegeneration. Environmental risk factors and PD-associated gene mutations act in series and/or in parallel pathways, likely sharing some common molecular mechanisms. Animal models of PD created by using individual neurotoxins or genetic manipulation have been extremely valuable for further studying the pathogenesis of PD. However, although available PD animal models reproduce many features of this disease, none of them replicates the full-spectrum of the disease. On the one hand, most of genetically engineered animals carrying PD-associated genetic defects failed to mimic the overt nigrostriatal dopaminergic neurodegeneration, suggesting that a single genetic risk factor is not sufficient to produce PD neurodegeneration. On the other hand, most of toxin-based PD models are generated by acute or subacute experimental exposure of young animals to relatively high doses of environmental toxins, and these models are often lack of classical α -synuclein pathology (e.g. abnormal α -synuclein aggregation and inclusion formation), a pathological hallmark of PD. There is tremendous hope that chronic progressive two-hit animal models involving both genetic mutations and environmental exposure will provide invaluable tool to advance our understanding of PD pathogenesis and to ultimately develop therapeutics that enable to retard PD progression. Clearly, additional animal studies as well as GWAS and epidemiologic studies aimed at identifying gene-environment interactions will glean many fresh insights into PD pathogenesis.

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Abbreviations

PD	Parkinson's disease
SN	substantia nigra
LPS	lipopolysaccharide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOS	nitric oxide synthase
LRRK2	Leucine-rich repeat kinase 2
PINK1	PTEN-induced putative kinase 1
GWAS	genome-wide association study
PET	positron emission tomography
GDNF	glial cell line-derived neurotrophic factor
SNPs	single-nucleotide polymorphisms

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Figure 1. PD develops from complex gene-environment interactions and involves multiple molecular pathways that crosstalk and converge leading to PD neurodegeneration

The identification of causative gene defects in PD has defined four major pathways leading to neuronal demise: protein aggregation, ubiquitin-proteasome system (UPS) dysfunction and impaired protein degradation, mitochondrial dysfunction and integrity impairment, and aberrant signal transduction. *SNCA* mutations and single-nucleotide polymorphisms (SNPs) make α -synuclein adopt a propensity for misfolding and accelerated aggregate formation. Excessive α -synuclein aggregates may overwhelm UPS protein degradation. Accumulated α -synuclein can translocate to the mitochondria and impair mitochondrial activity. *Parkin* mutations and *UCHL-1* SNPs prevent the proteolytic degradation of excessive toxic proteins (e.g. misfolded α -synuclein) in proteasomal machinery. *PINK1*, *Parkin*, and *DJ-1* functionally interact to maintain mitochondrial integrity and functionality and to protect cells against adverse effects of multiple stressors. Mutations in these genes cause mitochondrial dysfunction and subsequent decline in ATP production and increase in free radical generation, which results in oxidative stress and energy deficiency. Impaired mitochondria can release cytochrome c and other 'pro-apoptotic factors' triggering apoptotic cascades and cell death. Mitochondria in at least some forms of PD reveal abnormal morphology, impaired fission-fusion balance, and metabolic malfunction. *DJ-1* mutations reduce antioxidant response of cells, aggravating oxidative stress. Oxidative stress engages in diverse cellular processes and plays a prominent role in the induction of neuronal death. For instance, excessive production of free radicals can damage proteins (e.g. abnormal modification of α -synuclein and inactivation of Parkin), lipids, DNA, or RNA, leading to cell dysfunction (e.g. UPS and mitochondrial impairment) and eventual death. Mutations in *Pink1* and *LRRK2* induced aberrant kinase activity, altered substrate specificity, leading to inappropriate protein phosphorylation (e.g. increased α -synuclein phosphorylation at serine 129 by *LRRK2 in vitro*) and thereby affecting cell survival. Environmental toxins and brain trauma can trigger neuronal lesions by damaging mitochondria, causing oxidative stress, inducing inflammation in the central nervous system (CNS), and compromising defence mechanisms of cells. Some environmental risk factors can directly activate microglia (the resident immune cells in the CNS) or cause systemic inflammation, which in turn affects CNS inflammation. Genetic variation and polymorphisms in the *HLA* region and several inflammatory cytokines may become risk factors for PD. Activated microglia produce and secrete a spectrum of inflammatory and cytotoxic molecules, such as cytokines, chemokines, reactive free radicals, eicosanoids, and proteases. In addition to modulating microglial activity, these molecules influence the fate of surrounding neurons. Excessive inflammatory reaction usually becomes exaggerated and destructive, and turns into chronic inflammation that drives progressive neurodegenerative process. Injured neurons activate the surrounding microglia through the release or leakage of noxious self-compounds into the extracellular milieu, such as membrane breakdown products, abnormally processed or aggregated proteins (e.g. α -synuclein and β -amyloid), imbalanced neurotransmitters (e.g. elevated glutamate) and cytosolic compounds (e.g. α -synuclein, ATP, HMGB1 and neuromelanin). Thus, gene-environment interplay induces complex crosstalk among multiple signal cascades, forming a network and culminating in neuronal death and PD development.

Table 1

Human association studies identifying influence of gene-environment interactions on PD susceptibility

SNP	protein product of gene	environmental factors	references
<i>DAT</i>	dopamine transporter	pesticides	Ritz et al, 2009; Kelada et al, 2006
<i>NOS1 (nNOS)</i>	neuronal nitric oxide synthase	pesticides	Hancock et al, 2008
<i>NOS1 (nNOS)</i>	neuronal nitric oxide synthase	cigarette smoking	Levecque et al, 2003; Hancock et al, 2008
<i>NOS2A (iNOS)</i>	inducible nitric oxide synthase	cigarette smoking	Hancock et al, 2008
<i>APOE</i>	apolipoprotein E	caffeinated coffee	McCulloch et al, 2008
<i>SNCA</i>	α -synuclein	cigarette smoking	McCulloch et al, 2008
<i>GSTP1</i>	glutathione S-transferase pi 1	pesticide	Menegon et al, 1998
<i>CYP2D6</i>	cytochrome P-450 2D6	solvent exposure	De Palma et al, 1998
<i>GSTM1</i> null	glutathione S-transferase Mu 1	heavy solvent exposure	Dick et al, 2007