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Model Fusion: The Next Phase in Developing Animal Models for Parkinson's Disease

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Within the past 25 years, discoveries of environmental and monogenetic forms of parkinsonism have shaped the direction of Parkinson's disease (PD) research and development of experimental systems to study PD. In this review, we outline a remarkable array of *in vivo* models available, with particular emphasis on their benefits and pitfalls and the contribution each has made to enhance our understanding of pathological mechanisms involved in PD. Further, we discuss the increasingly popular approach of "model fusion" to create a new generation of animal systems in which to study gene-environment interactions, and the usefulness of such models in capturing the most common events underlying PD.

Keywords: Parkinson's disease; Animal modeling; MPTP; Transgenic animals; Genes; Environment

INTRODUCTION

The last few decades have witnessed an explosion of interest in Parkinson's disease (PD) research. This is in no small part due to the rapidly proliferating number of new disease models that are available. There is little doubt that these new models are making a major difference in our ability to study the cause of the disease, investigate underlying mechanisms of cellular degeneration, and explore new therapeutic approaches, including those directed at slowing or stopping disease progression. While we have had excellent ways to lesion the dopaminergic nigrostriatal system to model the disease in the past, such as the intracerebral administration of the dopaminergic neurotoxin 6-hydroxydopamine (Segura Aguilar and Kostrzewa, 2004; Kostrzewa *et al.*, 2006), the discovery of neurotoxins that selectively lesion this same area of the brain after systemic administration have given birth to new and more efficient ways to induce models for PD (Burns *et al.*, 1983; Heikkila *et al.*, 1984; Langston *et al.*, 1984), and opened up the possibility that similar compounds in the environment might actually play a causative role in the idiopathic disease itself. The second major impetus for the development of new models for PD has resulted from the identification of several different monogenetic forms of parkinsonism (for review, see Riess *et al.*, 2006) This began with discovery of a mutation in the gene that encodes for the protein α–synuclein (Polymeropoulos *et al.*, 1997), now known as the PARK 1 locus. This discovery not only demonstrated for the first time that a specific mutation can cause a well-defined form of parkinsonism, but it also lead to the identification of α–synuclein as a major component of Lewy bodies and neurites in both familial and sporadic PD. These observations

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have proved to be of enormous importance, providing a common thread for research on virtually all forms of Lewy body parkinsonism, which in turn has provided new experimental strategies for elucidating the mechanisms underlying neurodegeneration in these disorders. As expected, these and several other monogenetic forms of parkinsonism that have been identified since (discussed further below), have triggered a huge rush to develop transgenic mouse models based on the causative genes. Once again, these models have made major contributions to our current understanding of the mechanisms of cell death in PD, but virtually all of the transgenic models described to date have had shortcomings, not the least of which is a failure to exhibit degeneration of the nigrostriatal system, one of the key pathological features of PD.

In this article we review a remarkable array of new models that have been developed over the last several decades as a result of these discoveries, and summarize the pros and cons of each, with a focus on how they have contributed to a better understanding of PD. While both the genetic and neurotoxicant models all have their pluses and minuses, one fascinating, and even unexpected, outcome of these various lines of research is the way that they are being used in conjunction with each other in a highly synergistic fashion, bringing about new observations, discoveries and lines of research that would not have been possible with either the transgenic or neurotoxicant models alone. This new "fusion" of models is discussed in detail in the final section of this article because it is both novel and provides an entirely new set of tools in which gene-environment interactions can be studied experimentally. In the long run, combining these new tools may prove to be one of the most valuable when it comes to our quests to find the cause of the PD.

Part 1. Models of PD Induced by Systemic Exposure to Neurotoxicants

While theories on possible environmental causes of PD have waxed and waned over the last century and a half, interest in a environmental etiology of PD experienced a renaissance of interest with the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) induces an unalloyed parkinsonism in humans that is virtually indistinguishable from the idiopathic disease (Langston *et al.*, 1983). This toxicant, as well as others with similar structure (*i.e.*, the herbicide paraquat) or biochemical mode of action (*i.e.*, the pesticide rotenone), have been demonstrated to selectively induce degeneration of dopaminergic nigrostriatal neurons when administered systemically to various animal models. However, to date, no consistently reproducible toxicant exposure model fully recapitulates all the cardinal features of PD, such as the widespread Lewy body and neurite formation that is seen in the idiopathic disease or its *progressive* nature. However, there is little doubt that these exogenous exposure models have provided major insights into molecular mechanisms that underlie the process of neuronal degeneration, and have also provided clues that could lead to the elucidation of risk factors for PD. In this section we will focus on models of systemic toxicant administration that facilitate the death of dopaminergic neurons within the substantia nigra, but will not review models of intracerebral injections (*i.e.*, 6-OHDA) or terminal-specific injury (*i.e.*, methamphetamine) (for reviews on these models, see Guilarte, 2001; Fornai *et al.*, 2004; Segura Aguilar and Kostrzewa, 2004; Kostrzewa *et al.*, 2006; Mauceli *et al.*, 2006).

The MPTP Model of PD

How closely do the toxicant models come to mimicking the idiopathic disease in humans? The reality is that each model has it own strengths and weaknesses. Undoubtedly the MPTP model is the best studied to date, and is probably the closest to human PD when it comes to changes in behavior and neuronal circuitry in the basal ganglia. A consistent depletion of striatal dopamine and metabolites and degeneration of tyrosine hydroxylase-positive neurons in the substantia nigra are key pathological features of MPTP administration in a wide variety of species, but only primates demonstrate most if not all of the motor features of PD in an easily identifiable manner. Although inclusion body pathology has been reported in rodents and monkeys chronically exposed to the toxin (Forno *et al.*, 1986; Kowall *et al.*, 2000; Petroske *et al.*, 2001; Fornai *et al.*, 2005), the structure of the deposits do not recapitulate typical Lewy body morphology observed in sporadic PD brain. Even though replication of the non-motor PD features is

not consistently obtained, the MPTP model remains as the standard for evaluating nigral cell death and has allowed for highly focused research in a variety of areas such as, i) investigating mechanisms of degeneration (*i.e.*, mitochondrial complex I inhibition leading to oxidative damage) (Nicklas *et al.*, 1985; Ramsay and Singer, 1986; Richardson *et al.*, 2007), ii) the identification of features of dopaminergic neurons that contribute to their selective vulnerability (*i.e.*, the promiscuity of the dopamine transporter (DAT) (Javitch *et al.*, 1985), iii) defining synergistic interactions with other exogenous agents (*i.e.*, the fungicide diethyldiothiocarbamate (DDC) (Walters *et al.*, 1999) as well as endogenous compounds such as iron (Youdim and Riederer, 1993), and iv) providing new insights into toxicant-α–synuclein interactions during the process of nigral cell degeneration (Vila *et al.*, 2000).

Studies of MPTP toxicity have demonstrated that its bioactive metabolite, 1-methyl-4-phenylpyridinium (MPP^+) accumulates in mitochondria (Ramsay and Singer, 1986), directly interacts with complex I (Richardson *et al.*, 2007) and inhibits NADH dehydrogenase (Nicklas *et al.*, 1985), resulting in a dramatic reduction of intracellular ATP generation (Chan *et al.*, 1993) and the production of reactive oxygen species (ROS) (Cleeter *et al.*, 1992). These events may be toxic to any cell dependent on oxidative phosphorylation for energy production; and interestingly, decreased complex I activity has been demonstrated in substantia nigra from PD patients (Schapira, 1994). Taken together, these findings suggest that nigral dopaminergic neurons may be particularly vulnerable to complex I inhibition. Indeed, subsequent studies of complex I inhibition by the organic pesticide rotenone have demonstrated nigrostriatal injury in rodents, lending further support to this idea (Betarbet *et al.*, 2000).

A key feature of the MPTP model is that *selective* nigrostriatal toxicity is observed, similar to the pattern of cell loss in typical, sporadic forms of parkinsonism. Studies to determine the cause of MPTP-dopaminergic selectivity provided a number of insights into the mechanisms underlying the enhanced vulnerability of nigrostriatal neurons. Indeed, the selectivity of MPTP is conferred by the activity of a protein intrinsic to dopaminergic neurons, the dopamine transporter (DAT) localized in the plasma membrane. For MPTP toxicity to occur, the lipophilic molecule crosses the blood-brain barrier and is metabolized within astrocytes, by monoamine oxidase, to form MPP^+, the bioactive form of the toxin. Once the molecule is converted to its oxidized bipyridyl metabolite, the selective toxicity is conferred, as MPP^+ then enters neuronal cells via the DAT (Javitch *et al.*, 1985). Thus, dopaminergic neurons accumulate the toxic MPP^+ congener. At this point, cytosolic MPP^+ can be taken up by mitochondria (Ramsay and Singer, 1986), or alternatively, it can accumulate within synaptic vesicles as the result of active uptake by the vesicular monoamine transporter (VMAT2) (Daniels and Reinhard, 1988). Vesicular uptake of MPP^+ is thought be a neuroprotective event as it sequesters the metabolite away from its site of action, the mitochondrion; indeed, inhibition of VMAT2 *in vitro* potentiates MPP^+ toxicity, and transgenic mice with reduced VMAT2 expression are more susceptible to MPTP-induced nigrostriatal damage (Gainetdinov *et al.*, 1998). Thus, it has been hypothesized that the ratio of DAT to VMAT2 levels plays a critical role in mediating toxicity of exogenous agents (*i.e.*, MPTP) and endogenous molecules which promote intracellular oxidative damage (*i.e.*, dopamine) (Miller *et al.*, 1999).

Studies with the MPTP model also provided evidence to support the hypothesis that nigrostriatal toxicity can be mediated by synergistic mechanisms within dopaminergic neurons. In animal models of DDC exposure, no nigrostriatal injury is detected with the fungicide alone; however, when DDC administration precedes injection of MPTP, MPTP toxicity is dramatically potentiated (Walters *et al.*, 1999), indicating that exogenous agents may interact to elicit/enhance nigrostriatal damage. Further, this synergistic effect with MPTP is not exclusive to thiocarbamates such as DDC, but has also been noted in animal models of iron loading in which mice receiving a high iron diet were more susceptible to MPTP (Lan and Jiang, 1997). Studies of MPTP in monkeys have also demonstrated that iron accumulation may be a consequence of toxin exposure (Mochizuki *et al.*, 1994). Increased iron levels have been hypothesized to contribute to the generation of endogeneous 6-hydroxydopamine and toxic dopaminergic quinones (Pezzella *et al.*, 1997), and thus may further exacerbate the oxidative damage that occurs following MPTP exposure (Kaur *et al*., 2003).

The Rotenone Model

The potent effects of MPP^+ on mitochondrial Complex I quickly led to studies in patients with PD, showing similar deficits in brain and platelets (Parker *et al.*, 1989; Schapira *et al.*, 1990; Yoshino *et al.*, 1992). These observations in turn inspired Betarbet and colleagues (2000) to test the effects of another complex I inhibitor, rotenone. And indeed, consistent with the idea that nigral dopaminergic neurons display enhanced sensitivity to agents which elicit complex I inhibition, chronic systemic administration of the pesticide rotenone was found to produce nigrostriatal damage with α–synuclein- and ubiquitin-positive inclusion pathology in a subset of exposed rats with a dramatic motor behavioral phenotype (Betarbet *et al.*, 2000). In severely affected rodents, cell death within the substantia nigra occurred along with focal (*i.e.*, central region of maximal damage) or diffuse loss of TH-immunoreactivity in the striatum (Betarbet *et al.*, 2000; Hoglinger *et al.*, 2003; Sherer *et al.*, 2003; Fleming *et al.*, 2004; Lapointe *et al.*, 2004; Zhu *et al.*, 2004). Locomotor impairments were dose-dependent (Alam and Schmidt, 2002; Fleming *et al.*, 2004) and included dramatically reduced spontaneous activity, as well as dystonia and postural defects. Furthermore, variable degeneration has been reported, ranging from severe degeneration of dopaminergic neurons to no significant effect within the treated cohort of the same experiment (Betarbet *et al.*, 2000; Sherer *et al.*, 2003; Fleming *et al.*, 2004; Lapointe *et al.*, 2004; Zhu *et al.*, 2004; Hoglinger *et al.*, 2005). Interestingly, in less affected rats, motor deficits have been reported even though nigral neurons were spared (Zhu *et al.*, 2004) and striatal TH-immunoreactivity was not markedly altered (Fleming *et al.*, 2004). This may be attributed to the involvement of extranigral motor systems, as injury to non-dopaminergic striatal neurons and the globus pallidus has been observed in some studies of systemic rotenone exposure (Ferrante *et al.*, 1997; Hoglinger *et al.*, 2003). Similar results have been observed in mice where rotenone administration did not alter nigral dopaminergic cell density (Richter *et al.*, 2007), but caused motor deficits. Thiffault *et al.* (2000) have found that these behavior changes may be due to alterations in striatal dopamine neurotransmission in treated mice, such as enhanced dopamine turnover. Overall, this body of literature indicates that chronic, systemic administration of rotenone can produce nigrostriatal pathology, ranging from mild to robust, and thus may be a suitable model for both early and later stages of pathological mechanisms contributing to nigral cell degeneration.

The Effect of Paraquat on the Nigrostriatal System

Many groups of heterocyclic molecules with structural similarity to MPTP/MPP^+ have been proposed as possible environmental toxicants that might lead to PD, thus implicating them as potentially cytotoxic to dopaminergic neurons. Indeed, both epidemiological and case studies have indicated that other agents with bipyridyl structure such as diquat (Sechi *et al.*, 1992) and paraquat (Sanchez-Ramos *et al.*, 1987; Liou *et al.*, 1997) may contribute to the pathological events that lead to sporadic parkinsonism. For these reasons, a number of groups, including our own, have undertaken experiments to see if paraquat can be used to develop experimental models of PD. While earlier investigations on the effects of paraquat exposure in mice revealed no evidence of striatal dopamine depletion (Perry *et al.*, 1986; Widdowson *et al.*, 1996), subsequent studies have demonstrated clear-cut evidence of selective loss of dopaminergic neurons in the substantia nigra (Brooks *et al*., 1999; Peng *et al*., 2004), even though striatal dopamine levels remained within normal range (McCormack *et al.*, 2002). Further examination revealed enhanced activity of TH (the rate limiting factor in the synthesis of dopamine) in the striatum, thus suggesting that the discrepancy between nigral cell loss and this marker of striatal injury (*i.e.*, reduced dopamine) in the systemic paraquat model is likely due to this compensatory response of the still intact nigrostriatal terminals (Thiruchelvam *et al.*, 2003). Similar to MPTP-induced injury, paraquat neurotoxicity is enhanced when administered in combination with the thiocarbamate, MANEB (Thiruchelvam *et al.*, 2000) as well as other toxins (Shepherd *et al.*, 2006).

Although structurally similar to MPP^+, paraquat does not exert its selective dopaminergic toxicity through DAT-mediated uptake (Richardson *et al.*, 2005). While the mechanism of neuronal paraquat uptake remains poorly understood, its passage through the blood-brain barrier is mediated, at least

in part, via the neutral amino acid transporter, the same carrier mechanism utilized for transport of levodopa (Shimizu *et al.*, 2001; McCormack *et al.*, 2003). This fascinating observation raises the important question as to whether or not this system may provide an entry point for exogenous neurotoxicants to gain access to the CNS.

There are several other differences between the effect of bipyridyls such as paraquat and MPP^+. Once in the brain, MPP^+ is taken up into dopaminergic neurons by the dopamine uptake system, at least in part, explaining its selectivity for this system. However, paraquat is not a substrate for the DAT, and the reasons for its selective effects on the nigrostriatal dopaminergic system remain largely unknown. Another discrepancy between the bipyridyls such as paraquat and MPP^+ relates to their mechanisms of action; paraquat does not bind and subsequently inhibit complex I activity at levels that are physiologically relevant (Richardson *et al.*, 2005). In contrast, recent studies have revealed that paraquat administration leads to the production of ROS through a redox cycling mechanism; indeed, NADPH oxidase and nitric oxide synthase localized on microglia appear to trigger paraquat redox cycling and ROS production (Bonneh-Barkay *et al.*, 2005). Interestingly, in the mouse model of subchronic paraquat exposure, repeated injections are required to elicit significant oxidative damage and dopaminergic degeneration (Purisai *et al.*, 2007), as one systemic dose of paraquat is not sufficient to induce cell death. Rather a single injection seems to enhance the vulnerability of dopaminergic neurons to subsequent exposures, acting as a "priming" event. The sequence of events required for paraquat toxicity via this model is emerging, the first paraquat injection activates microglia, which in turn bioactivate and trigger redox cycling of paraquat when subsequent injections of the toxin are administered. This hypothesis is supported by a study using the potent anti-inflammatory minocycline. When minocycline was administered prior to paraquat, subsequent insults failed to elicit oxidative stress and neurodegeneration, demonstrating the requirement for the inflammatory response in paraquat-mediated toxicity (Purisai *et al.*, 2007). Thus, microglial activation by paraquat, or other agents such as lipopolysaccharide (LPS) (see next section), may enhance the vulnerability of dopaminergic cells to toxicant-induced damage.

The Effects of LPS on the Nigrostriatal System

A solid body of literature is emerging from toxicant exposure models (as well as analyses of human brain) that implicates a role for inflammation in the cascade of degenerative events underlying nigral dopaminergic cell death. Indeed, recent *in vivo* studies of systemic LPS exposure have begun to elucidate the mechanisms which contribute to the inflammatory response in the substantia nigra and the long-term consequences of glial activation. In an interesting series of experiments, Ling *et al.* (2004) have shown that administration of a single LPS treatment to pregnant dams at E10.5 results in reduced dopaminergic neurons in pups at 10 days and 3 weeks following birth. Prenatal exposure to LPS also rendered adult rodents more vulnerable to nigrostriatal injury by 6-OHDA, indicating a synergistic effect between inflammation and toxicant administration at a later time, indicating the long-term latency effect of the inflammatory response. Subsequent studies by this same group have provided insights into pathways that contribute to latency, revealing that increased levels of microglia are present in animals that received prenatal treatment with LPS (Ling *et al.*, 2006). In a fascinating new report by Qin *et al.*, a single LPS injection to adult mice elicited an increase in production of the cytokine TNF-α that persisted in the brain for 10 months, although levels in the periphery returned to normal within a week (Purisai *et al.*, 2007). Furthermore, at 7 months following LPS administration, the number of tyrosine hydroxylase-positive neurons in the substantia nigra was reduced by approximately 25%, and interestingly, progressed to nearly 50% by 10 months. Taken together, these findings show that a single exposure to LPS in adult mice, (i) can trigger increased release of inflammatory cytokines via activation of microglia that is sustained in the brain, and (ii) eventually results, over time, in loss of nigral dopaminergic neurons which appears to be progressive. A fascinating feature of this model, which for the moment at least represents a "black box" in knowledge, is the several month time delay between administration of LPS and appreciable nigrostriatal injury. This finding suggests that there can be an extended time delay between an insult and its toxic consequences; we

have previously referred to this as a "long-latency" toxicity, a concept that is of obvious importance to the study of neurodegenerative disease (Purisai *et al.*, 2007). Further, the selectivity of toxicity suggests that other processes intrinsic to dopaminergic neurons in the substantia nigra may contribute, over time, to pathogenesis.

Taken together, these results support the idea that "multiple hit" models may be required to recreate the most, if not all of the cardinal features of PD, and further, suggest that pre-existing (and usually silent) conditions such as glial activation (or α–synuclein upregulation - see next section), may synergistically interact with toxicant exposure to produce nigrostriatal injury.

Part 2. Transgenic Mouse Models

The race to develop transgenic models for PD began in earnest after of the identification of the causative mutation for a monogenetic form of familial parkinsonism (Polymeropoulos *et al.*, 1997). Polymeropoulos and colleagues (1997) demonstrated the presence of missense mutations in the gene for α–synuclein in a kindred with early-onset autosomal dominant parkinsonism (now referred to as PARK 1); subsequently, two additional causative mutations have been identified in the gene for α–synuclein (Kruger *et al.*, 1998; Zarranz *et al.*, 2004). Since that time, mutations have been identified in three genes that cause autosomal recessive parkinsonism, parkin (*PARK 2*), DJ-1 (*PARK 7*) and PINK-1 (*PARK 6*) (Kitada *et al.*, 1998; Bonifati *et al.*, 2003; Valente *et al.*, 2004). More recently another form of autosomal dominant parkinsonism has been identified, which is due to mutations in the gene for LRRK2 (PARK 8) (Paisan-Ruiz *et al.*, 2004; Wintermeyer *et al.*, 2000). Additionally, sequence variations in several other proteins linked to development and maintenance of nigrostriatal neurons have been implicated as susceptibility genes (*i.e.*, Nurr-1 and tau; Ramsden *et al.*, 2001; Zhang *et al.*, 2005; Fung *et al.*, 2006). As noted above, the use of transgenic models has been aggressively employed as a way to elucidate the cellular functions of affected proteins and their relevance to mechanism involved in degeneration, and to determine whether their respective mutations can elicit pathology reminiscent of PD. However, similar to the neurotoxicant models, none of these genetically engineered mice have delivered "the magic bullet" by providing a model with typical motor and non-motor features of PD, progressive nigrostriatal degeneration manifested by striatal dopamine depletion and nigral cell death, and the presence of α–synuclein-positive Lewy bodies and Lewy neurites. However, again, similar to the neurotoxicant models, transgenic mice models have served the critical purpose of providing new insights into the biological relevance of these proteins, both in health and disease.

Models Based on α–Synuclein

In addition to the missense mutations in the gene for α–synuclein reported by Polymeropoulos (A53T mutation; 1997), Kruger (A30P mutation; 1998) and Zarranz (E46K mutation; 2004), genomic duplication and triplication of wildtype α–synuclein (Farrer *et al.*, 2004; Singleton *et al.*, 2003; Chartier-Harlin *et al.*, 2004; Fuchs *et al.*, 2007) have subsequently been detected in autosomal dominant PD families. The importance of the discovery that multiplications of the gene for normal α–synuclein can lead to the full spectrum of Lewy body pathology cannot be overestimated, as it shown for the first time that too much non-mutated α–synuclein can cause virtually all of the pathology seen in the sporadic disease, pointing to a role for native α–synuclein in the neurodegenerative process itself. The pathology associated with the mutations/multiplications is not wholly consistent, however, ranging from nigral dopaminergic cell death with cytoplasmic Lewy body formation, to the presence of neuritic and perikaryal tau inclusions throughout the brain (Spira *et al.*, 2001; Duda *et al.*, 2002). Furthermore, differences in the clinical phenotype have been observed. For example, the triplications are more reminiscent of dementia with Lewy bodies, whereas patients with duplications of the α–synuclein gene are said to have a later onset and with a slower disease course (Chartier-Harlin *et al.*, 2004) that is more typical of sporadic disease. It is important to note, though, that mutations in α–synuclein are extremely rare and represent only a small percentage of familial PD cases. However, the identification of causative mutations in the gene encoding for α–synuclein proved to have much broader implications. Specifically, α–synuclein is

the major structural component of Lewy bodies and dystrophic neurites, the pathological hallmarks of disease, in not only brain tissues from α–synuclein kindreds, but also *idiopathic* cases (Spillantini *et al.*, 1998), marking this protein as a critical player in the pathophysiology underlying familial and sporadic PD.

To elucidate how α–synuclein contributes to the demise of nigral dopaminergic neurons and other pathological features of PD, a variety of α–synuclein transgenic mice have been generated, varying in tissue-specificity (promoter), mutation (A53T, A30P, WT and truncation) and expression levels (over-expression or knockout). One of the main lessons that has been learned, as might have been expected, is that the effects of α–synuclein alteration on the nigrostriatal function, inclusion pathology and behavior are dependent on the expression level of the protein and tissue-specificity of the promoter.

The PDGF-B Promoter: The first description of an α–synuclein over-expressing transgenic was published by Masliah and colleagues (2000). This transgenic mouse over-expressed human wild-type α–synuclein (H-α–syn) under the control of the PDGF-B promoter. While pathological analyses of these mice did not demonstrate a complete recapitulation of the cardinal features of PD, the findings were impressive and included (i) a progressive reduction in TH-immunoreactivity in the striatum, (ii) locomotor impairment and (iii) the presence of α–synuclein-positive cytoplasmic deposits of a granular nature and larger intranuclear inclusions containing dense cores. However, intranuclear inclusions are not typically seen in PD, and in their model no nigral dopaminergic cell loss was detected, suggesting that α–synuclein over-expression alone is not sufficient to cause nigral cell degeneration, at least in the mouse. Thus, the strength of this model is the effect of PDGF-driven H-α–syn over-expression on the dopaminergic nigrostriatal terminal fields, replicating at least one aspect of PD. This is important in view of the increasing recognition that α–synuclein misfolding/aggregation may cause significant neuronal dysfunction regardless of whether or not there is cell loss, and there is no doubt that this aspect of PD deserves intensive investigation.

The Thy-1 Promoter: Following recognition of the strengths and weakness of the PDGF-B H-α–syn over-expressing mice, other lines of transgenics were developed to explore the effects of different α–synuclein expression levels to determine if they would lead to pathological features more similar to that seen in PD. α–Synuclein over-expression, driven by the mouse Thy-1 promoter (mThy-1) was found to induce high levels of the protein in several brain regions including striatum, substantia nigra, thalamus and brainstem, as well as the spinal cord (Kahle *et al.*, 2000a,b; van der Putten *et al.*, 2000; Neumann *et al.*, 2002; Rockenstein *et al.*, 2002). In line with the concept that α–synuclein plays a role in synaptic maintenance, over-expression of both wild-type and mutant human α–synuclein promoted abnormal accumulation in synaptosomes/terminal fields and neurites, pathologically presenting as swollen axonal varicosities (Kahle *et al.*, 2000a). These pathological features were accompanied by severe, progressive locomotor deficits reported in one line of mThy-1-driven human A53T α–synuclein over-expression (van der Putten *et al.*, 2000). However, the pathological changes were observed predominantly in the brainstem, spinal cord and the neuromuscular junction, but not in the substantia nigra, where there was no α–synuclein (van der Putten *et al.*, 2000). A decline in motor function, astrogliosis and formation of proteinase K-resistant α–synuclein deposits has been detected in transgenic mice overexpressing the A30P mutant form of the protein; yet again, the primary target of PD modeling, the substantia nigra was spared in these animals, with the sensorimotor nuclei within the brainstem and spinal cord bring primarily affected (Neumann *et al.*, 2002).

A mouse-Thy-1 H-α–syn transgenic line was described by Rockenstein and colleagues (2002) that does exhibit nigral accumulation of the protein, with diffuse cortical expression as well. Along with locomotor impairment, these mice also displayed progressive deficits in sensorimotor behaviors detectable as early as 2 months of age, indicating that behavioral phenotypes due to alterations in nigrostriatal transmission may be present prior to the development of discernable pathology (Fleming *et al.*, 2004). Overall, the consistent finding among these studies is that α–synuclein over-expression using the Thy-1 promoter leads to accumulation of

the protein within cell bodies, early neuritic pathology and a behavioral phenotype that worsens with age, indicating that it will be useful to study the effects of α–synuclein on neuronal function and possibly disease progression over time. The neuropathological changes, however, were typically observed in regions other than the substantia nigra, and in non-neuronal cells of glial morphology. While not replicating the selectivity of PD *per se*, these observations implicate α–synuclein as possibly having a role in a wide range of cellular pathologies, including multiple system atrophy, which have been coined collectively as "α–synucleinopathies" (Galvin *et al.*, 2001).

The PrP Promoter: Multiple lines of transgenic mice over-expressing wildtype, A30P or A53T α–synuclein proteins have been generated under the control of the prion promoter (PrP; Giasson *et al.*, 2002; Lee *et al.*, 2002; Gispert *et al.*, 2003; Gomez-Isla *et al.*, 2003); some of the most severe behavioral impairments, leading to frank paralysis and death, have been reported in lines of mice with PrP-driven A53T over-expression (Giasson *et al.*, 2002; Lee *et al.*, 2002; Gispert *et al.*, 2003). Reminiscent of Thy-1-driven expression, abnormal α–synuclein accumulation in these transgenic lines occurs in several brain regions including cortex and amygdala, but predominantly in the motor nuclei of brainstem and spinal cord (Giasson *et al.*, 2002; Lee *et al.*, 2002; Gispert *et al.*, 2003), and motor impairments have been generally attributed to degeneration within these pathways as opposed to the substantia nigra. Indeed, a robust loss of motor neurons in the spinal cord (75%) was reported in PrP-h A53Tsyn mice along with markers of mitochondrial DNA damage and apoptosis, suggesting that A53T over-expression contributes to oxidative stress and proapoptotic mechanisms within these cells (Martin *et al.*, 2006). These findings have led to the suggestion that α–synuclein-related mechanisms may contribute to pathogenesis of motor neuron disease (although clinical observations have yet to support this hypothesis) and that this model, albeit extra-nigral, provides a system to gain insight into the cellular pathways involved in mediating α–synuclein toxicity. Thus, while the behavioral and pathological selectivity differ substantially from that seen in PD, this model is still being used to study mechanisms of neuronal degeneration. Furthermore, it could be argued that the cortical changes, which are also seen in these animals, might be used to model Braak Stage 5 or 6, which occurs in advanced PD.

The TH Promoter: To test the contribution of enhanced α–synuclein levels to nigrostriatal toxicity, lines of transgenic mice have been generated with protein over-expression targeted for catecholaminergic neurons using the TH promoter (Matsuoka *et al.*, 2001; Rathke-Hartlieb *et al.*, 2001; Richfield *et al.*, 2002). Although α–synuclein accumulation occurred with nigral neurons, mice expressing human wildtype or A53T or A30P mutation alone did not demonstrate alterations in the number of TH-immunoreactive neurons in the substantia nigra, nor were there changes in striatal dopamine and metabolite levels (Matsuoka *et al.*, 2001; Rathke-Hartlieb *et al.*, 2001; Richfield *et al.*, 2002). Mice expressing double human α–synuclein mutations (*i.e.*, both A53T and A30P) did reveal an age-related reduction in striatal dopamine accompanied by locomotor impairments (Richfield *et al.*, 2002) and nigral dopaminergic cell loss (Thiruchelvam *et al.*, 2004). Ironically, robust α–synuclein pathology was not induced by over-expression in the neurons thought to be sensitive to its toxicity. However, recent studies of TH-driven over-expression of truncated forms of α–synuclein have revealed alterations in the nigrostriatal system (Tofaris *et al.*, 2006; Wakamatsu *et al.*, 2006). Tofaris *et al.* (2006) have reported that over-expression of truncated α–synuclein 1-120 on an α–synuclein null background leads to reduced striatal dopamine concentrations (by about 30%), microglial activation in the substantia nigra, deposition of α–synuclein and progressive behavioral defects. Decreased striatal dopamine concentrations and locomotor impairments have been reported in another set of studies utilizing transgenics that express human α–synuclein 1-130 under the control of the rat TH promoter on a C57BL/6 background (Wakamatsu *et al.*, 2006). While no evidence of α–synuclein aggregation or inflammation was detected in this line, mice were born with reduced numbers of TH-positive neurons in the substantia nigra compared to wildtype littermates (Wakamatsu *et al.*, 2006). It is unclear if this is a developmental

phenomenon, and there is actually neuronal degeneration *in utero*, as there is no further loss of cells after birth. Overall, the findings from these two groups implicate truncated α–synuclein protein in nigrostriatal degeneration and suggest that differential α–synuclein toxicity may be dependent on the isoform expressed. Interestingly, one of these transgenics also exhibited clear-cut α–synuclein pathology in the olfactory bulb. This is potentially important, as it may provide an opportunity to model Braak Stage 1 PD, which is characterized by early involvement of the olfactory bulb, as well as the dorsomotor nucleus of the vagus.

In summary, for transgenic model based on TH-driven α–synuclein over-expression, a spectrum of toxicity exists, selective nigrostriatal injury (*i.e.*, reduced nigral cell counts) has been documented only in lines overexpressing double mutant α–synuclein or a truncated form (Thiruchelvam *et al.*, 2004; Wakamatsu *et al.*, 2006), whereas in other lines, no overt nigral pathology is detectable (Matsuoka *et al.*, 2001; Rathke- Hartlieb *et al.*, 2001). Indeed, the lack of injury in some of these models suggests that compensatory -- and possibly neuroprotective-mechanisms exist within α–synuclein overexpressing transgenics, raising the possibility that the potential of these models might be fully realized only in the context of one or more toxicant exposures. In this scenario, nigral α–synuclein expression could be used experimentally as a risk factor of nigral cell degeneration. This in turn may provide an new experimental paradigm in which these "at risk" but "unaffected" mice can be used as tools to study the effects of multiple hits on the nigrostriatal pathway and thereby model gene-environment interactions.

Models Based on Parkin (PARK 2)

The parkin protein is a 465-amino acid E3 ubiquitin ligase that targets specific substrates, including glycosylated α–synuclein, for degradation through the proteasome pathway. In 1998, Kitada and colleagues (1998) found that mutations in the gene for parkin were linked to autosomal recessive juvenile parkinsonism. Since then, over 100 mutations in parkin have been reported, and these mutations appear to represent the most common form of inherited early onset parkinsonism described to date. Interestingly, although diminished parkin function should promote the intracellular accumulation of proteins, Lewy body pathology is typically not a feature of parkinsonism caused by mutations in parkin (Hayashi *et al.*, 2000; Farrer *et al.*, 2001; Sasaki *et al.*, 2004; Pramstaller *et al.*, 2005) and the pathology (cell loss) is largely restricted to the substantia nigra.

While *parkin*-deficient mice have been generated, another mouse line with spontaneous motor behavior dysfunction, the *quaking viable* mouse, was discovered to have deletions in parkin and the parkin modifier, PACRG. In transgenic lines with detectable motor deficits, including an exon 3 deletion mouse described by Goldberg *et al.* (2003) and the quaking viable animals reported by Lockhart and colleagues (2004), increased extracellular levels of dopamine or its metabolite DOPAC have been detected. Interestingly, decreased mitochondrial function has been reported in at least one line of *parkin*-deficient mice, along with evidence for oxidative damage (Palacino *et al.*, 2004). However, no loss of dopaminergic neurons appears to occur in young or aged (*i.e.*, 24 months) in any of the parkin transgenic lines reported to date. Despite the lack of overt nigral pathology, subtle changes in locomotor (*e.g.*, spontaneous motor and beam challenge activities) and non-motor (*e.g.*, somatosensory adhesive removal and reduced exploratory) behaviors have been consistently detected (Goldberg *et al.*, 2003; Itier *et al.*, 2003). Interestingly, in one line of parkin knockouts, although the nigral neurons were spared, TH cell loss was detected in the locus coeruleus (Von Coelln *et al.*, 2004), a region of the brain which is almost always affected in PD (German *et al.*, 1992), but not typically in the few autopsied cases of parkin parkinsonism described to date.

Models Based on DJ-1(PARK 7)

DJ-1 is a 189-amino acid protein that is expressed in several brain regions including the substantia nigra (Nagakubo *et al.*, 1997; Shang *et al.*, 2004). This protein was linked to PD in a report describing two homozygous mutations in the DJ-1 gene in families with early-onset, autosomal recessive parkinsonism, a Leu-Pro substitution at position 166 in an Italian family and a deletion in exons 1-5, which includes the promoter start site for DJ-1, in a Dutch kindred (Bonifati *et al.*, 2003). The dele-

tion in the Dutch family results in a complete lack of DJ-1 expression and the point mutation changes the secondary structure such that its function is likely impaired, and further, promotes its catalysis through the ubiquitin-proteasome system (Moore *et al.*, 2003); thus, a loss of function of this protein appears to be the common feature in these two kindreds (Bonifati *et al.*, 2003). At the time of this writing, the neuropathological features of parkinsonism due to DJ-1 mutations in human remains unknown, as no autopied cases have been reported to date. Although the pathogenic mechanism by which a DJ-1 deficiency leads to parkinsonism has yet to be established, DJ-1 has been reported to be multifunctional, it has the properties of an antioxidant, can regulate transcription, serve as a chaperone, and may play a role in apoptosis (Bandopadhyay *et al.*, 2004; Canet-Aviles *et al.*, 2004; Shendelman *et al.*, 2004; Taira *et al.*, 2004; Xu J *et al.*, 2005; Zhou and Freed, 2005; Zhou *et al.*, 2006).

To date, three lines of DJ-1 transgenics have been reported. Similar to other transgenics of autosomal recessive PD mutations, loss of DJ-1 does not appear to cause nigral cell death, striatal dopamine depletion or the formation of α–synuclein inclusion bodies, even in mice up to 12 months of age (Chen *et al.*, 2005; Goldberg *et al.*, 2005; Kim *et al.*, 2005). However, changes in striatal dopamine reuptake and subtle, yet consistent behavioral impairments have been reported, and implicate increased activity of the DAT as a key to the observed alterations in nigrostriatal dopaminergic neurotransmission (Chen *et al.*, 2005; Goldberg *et al.*, 2005). Consistent with this hypothesis, enhanced striatal dopamine turnover occurs as a function of aging in these mice along with increased markers of striatal oxidative damage (Chen *et al.*, 2005). It is not yet clear how these changes will inform us regarding typical, sporadic disease other than suggesting we should concentrate more on the DAT itself, and or the potential roles of chaperone proteins and apoptosis.

PINK-1 (PARK 6) and LRRK2 (PARK 8)

At the time of this writing, neither PINK-1 nor LRRK2 transgenics have been reported in the literature. In regard to PINK-1, one might speculate that nigral cell loss will not be detected, given what is known regarding transgenics for other autosomal recessive mutations. On the other hand, it seems likely that subtle alterations to the nigrostriatal dopaminergic pathway will be found that could lend insights into factors that affect neuronal vulnerability. However, in the event that features typical of human PD are recapitulated in PINK-1-deficient mice, such a model would of course represent a major addition to our current armamentarium.

The jury is also still out when it comes to the phenotypic picture in LRRK2 transgenic mice, since none have yet been reported. If history is a guide, we may not see dramatic changes, although it can hoped that a more robust pathological picture will be encountered since the majority of pathological cases studied to date exhibit neuropathologic features that are virtually identical to typical, idiopathic Lewy body PD. However, it should also be noted that with several of the pathogenic mutations in this gene, considerable variability in pathologic phenotype has been documented, including one family that presented four different pathologic phenotypes in four different members, these included pure nigral cell loss, a second case with a progressive supranuclear palsy-like picture, and third with a phenotype resembling that seen in dementia with Lewy bodies, and a fourth with classic Lewy body pathology typical of idiopathic PD (Zimprich *et al.*, 2004; Rajput *et al.*, 2006). For the moment, we must wait until reports begin to appear on the behavioral, neurochemical, and pathological phenotypes in LRRK 2 transgenic mice.

Part 3. Model Fusion: Searching for New Insights into PD by Combining Neurotoxicant and Transgenic Models

As outlined in detail above, the overwhelming majority of transgenic mouse lines do not demonstrate overt damage to the dopaminergic nigrostriatal system, but rather have subtle changes that are likely relevant as risk factors, such as altered dopaminergic neurotransmission. On the other hand, the neurotoxicant models described do lead to degeneration of the dopaminergic cells in the substantia nigra. These observations have led a number of investigators to the obvious conclusion that combining these various models might represent a highly productive next step in the race to develop new and better models for PD. It is also clear that combining these very different models might provide insights

into mechanisms of neurodegeneration that could be gained in no other way.

To date, at least two different strategies have been employed. One approach is to examine the effects of a particular neurotoxicant on one or more of the proteins identified through human genetics described above. Such studies can be carried out in a variety of different biological systems.

Effects of Systemically Administered Neurotoxicants on α–Synuclein, Parkin, and DJ-1Proteins: Results of in vivo Studies

α–Synuclein: In a curious reversal of the usual sequence of carrying out *in vivo* investigations in rodents before going to higher species, the first attempt to determine the effects of MPTP on endogenous synuclein was actually performed in baboons (Kowall *et al.*, 2000). In this study, in addition to the expected nigral cell degeneration, MPTP induced intraneuronal accumulation and aggregation of α–synuclein, strongly suggesting a relationship between α–synuclein and MPTP exposure. Vila and colleagues (2000) subsequently explored the effects on MPTP on endogenous α–synuclein in rodents. These investigators used a regimen of MPTP exposure that was previously shown to elicit apoptotic changes in dopaminergic neurons in mice, and found that MPTP induced an increase of both α–synuclein mRNA and protein levels in the ventral mesencephalon. Somewhat surprisingly, however, this increase was found to occur only in TH-positive nigral cells that were not expressing markers of apoptosis. They concluded that in this model, at least, α–synuclein modulates the neurotoxic effects of MPTP and may be part of an effort on the part of the cell compensate for the effects of a toxicant challenge. More recently, studies in our own laboratory have shown that in non-human primate models of MPTP, exposure clearly increased expression of α–synuclein within nigral neurons including neuromelanin-positive cells, indicating this event occurs within dopaminergic neurons (Purisai *et al.*, 2005). Remarkably, in the primate model, both mRNA and protein levels remain elevated compared to controls, for at least one month after a single injection of the MPTP, indicating that a prolonged increase in α–synuclein expression can be induced by an exogenous neurotoxic exposure, in this case by MPTP. Furthermore, nigral cell death during this period was found to be progressive (from 10% at 1 week to 40% at 1 month), suggesting that a relationship may exist between sustained α–synuclein expression and neurotoxicity within the substantia nigra (Purisai *et al.*, 2005). We hypothesized that these changes could be used to model early stages in the pathological events leading to Lewy body formation. If so, evaluation of α–synuclein-MPTP interactions may provide a means by which to elucidate the processes involved in intracellular inclusion formation in PD.

Expression of α–synuclein is also up-regulated in dopaminergic neurons following paraquat exposure (Manning-Boğ *et al.*, 2002). In this model, the protein is translocated to cell nuclei of nigral neurons and co-localizes with acetylated histones (Goers *et al.*, 2003). Importantly, subsequent studies in Drosophila have correlated neurotoxicity with α–synuclein nuclear translocation and histone association (Kontopoulos *et al.*, 2006), providing further support for the idea that histone-α–synuclein interactions represent a potential mechanism by which α–synuclein contributes to degenerative events. Elevated levels of α–synuclein also appear to be an integral part of rotenone-mediated toxicity. Betarbet and colleagues (2006) have shown that chronic exposure to the pesticide in rodents causes accumulation and aggregation of α–synuclein, as well as reduced proteasomal function within the substantia nigra. This decline in proteasomal function could also be attributable to intracellular inclusion formation (Betarbet *et al.*, 2000).

Given the increasing evidence for the near ubiquitous involvement of α–synuclein in a variety of toxic events, it is not surprising that dopaminergic injury induced by LPS has been shown to be accompanied by an apparent increase in α–synuclein within nigral neurons (Zhang W *et al.*, 2005). Interestingly, these changes are similar to exposure paradigms utilizing toxicant known to produce oxidative injury (Vila *et al.*, 2000; Manning-Boğ *et al.*, 2002; Sherer *et al.*, 2003). The role of α–synuclein up-regulation following toxicant insult is not well understood; however, it is possible that increased levels of the protein may have a dual role, being involved in a compensatory response to help cells protect against neurotoxic insult, but also, depending on the species and circumstances, bestowing

enhanced susceptibility and possibility of becoming a part of the cascade of events that lead to neurodegeneration.

Other Proteins: Other proteins linked to monogenic forms of PD also reveal interesting alterations in normal animals after toxicant exposure. Modification of parkin has been noted in mice exposed to MPTP. These changes include S-nitrosylation of the protein and a reduction of its ubiquitin E3 ligase activity (Chung *et al.*, 2004b). Interestingly, this feature has also been demonstrated in PD brain, raising the possibility that similar alterations in parkin might be important in the process of nigral cell degeneration in the disease itself (Chung *et al.*, 2004a). Enhanced oxidation of DJ-1 has also been observed in rodents after chronic rotenone-treatment, again suggesting that there are important interactions between these various proteins and the mechanisms of action of various parkinsonogenic neurotoxicants (Betarbet *et al.*, 2006).

In summary, it seems that further evaluation of parkin, DJ-1, PINK-1 and LRRK2, as well as α–synuclein in these neurotoxicant models is likely to be highly informative, may be very helpful in deciphering the interaction or cross-talk between their respective mechanisms of action and are likely to provide invaluable information in our quest to elucidate the sequence of events that underlie the process of neurodegeneration.

The Effects of Systemically Administered Neurotoxicants on Transgenic Models of PARK 1, 2, & 6

α–*Synuclein-MPTP:* Given that the majority of transgenic mice being used to study PD are over-expressers of different forms (*i.e.*, mutations and truncations) of α–synuclein, it is not surprising that this model has been the most widely studied when it comes to exposing transgenic models to neurotoxicants. At least three different groups have reported increased sensitivity to MPTP using a variety of outcome measures including number of TH-immunoreactive neurons in the substantia nigra and striatal dopamine concentrations; these observations have been made in mice over-expressing both wild type and mutant forms of the human protein as compared to littermates (Richfield *et al.*, 2002; Song *et al.*, 2004; Nieto *et al.*, 2006). The results of these studies support the hypothesis that elevated levels of α–synuclein are likely to increase the susceptibility of the nigrostriatal pathway to environmental toxins. In one study (Song *et al.*, 2004), the development of α–synuclein aggregate pathology following sub-chronic MPTP exposure occurred only in α–synuclein transgenic mice. These mice also showed more extensive mitochondrial damage, axonal degeneration, and the formation of neuritic and cytoplasmic perinuclear inclusions (Song *et al.*, 2004). Abnormal PBS- and detergent-soluble aggregates of α–synuclein (of varying molecular weight between treatment and control groups) have also been isolated from transgenic and wildtype animals administered MPTP (Dalfo *et al.*, 2004), suggesting that these aggregates may be toxic α–synuclein congeners formed as a consequence of environmental exposure.

However, not all investigators have been able to confirm that increased levels of α–synuclein exacerbate the toxic effects of MPTP. In two separate lines of α-synuclein overexpressing mice in which accumulation of the protein in cell bodies and neurites was clearly evident (Rathke-Hartlieb *et al.* 2001), there were no differences between transgenics and littermate controls in dopaminergic cell number and dopamine concentration in the striatum after exposure to MPTP. However, it should be mentioned that in these two lines, there were no adverse effects of α–synuclein over-expression on the nigrostriatal pathway. Therefore it may be that for the additive effects of MPTP to be manifest, there must be at least some degree of cellular dysfunction present to serve as a risk factor for damage after toxic exposure.

Perhaps the most compelling evidence favoring a role for α–synuclein in toxicant-induced neurodegeneration comes from transgenic mouse lines that are deficient in α–synuclein expression. Depending on the regimen of MPTP employed, α–synuclein knockout animals have been found to be partially or fully resistant to the effects of MPTP (Dauer *et al.*, 2002, Schluter *et al.*, 2003; Drolet *et al.*, 2004; Klivenyi *et al.*, 2006). The mechanism underlying this extremely interesting set of observations is still far from clear, but it certainly deserves further investigation. One hint as to the mechanism has come from the study by Dauer *et al.* (2002),

in which they found that the ability of MPTP to inhibit complex I activity was dramatically reduced in α–synuclein knock-out animals, indicating some type of relationship between the protein and mitochondrial function.

α–*Synuclein-Paraquat:* Reports of exposure of α–synuclein transgenics to the herbicide paraquat are reminiscent of the MPTP-transgenic studies, in that they have already provided new insights into mechanisms of dopaminergic nigrostriatal degeneration, but have also led to some surprising results. As noted earlier, TH-driven α–synuclein over-expressing mice typically do not demonstrate degeneration of dopaminergic nigrostriatal neurons. We therefore hypothesized that increased α–synuclein would increase the vulnerability of nigral neurons to the toxic effects of paraquat (Manning-Boğ *et al.*, 2002). Favoring this hypothesis was the observation that, similar to MPTP, paraquat up-regulates expression of synuclein within nigrostriatal dopaminergic neurons in normal mice after systemic exposure (Manning-Boğ *et al.*, 2002). Unexpectedly, however, instead of enhanced toxicity, nigral neurons in these transgenic animals were partially protected against the neurotoxic effects of paraquat (Manning-Boğ *et al.*, 2003). Subsequent analysis has demonstrated that there is increased expression of the molecular chaperone HSP70 in TH-α–synuclein transgenics compared to wild-type controls, raising the possibility that α–synuclein over-expression is inducing this chaperone response which contributes to the amelioration of nigral paraquat toxicity (Manning-Boğ *et al.*, 2003). Indeed, a neuroprotective role for synuclein has subsequently been reported in another set of experiments using mice lacking the co-chaperone CSPα (Chandra *et al.*, 2005); CSPα deficiency promotes progressive neurodegeneration in mice, suggesting the chaperone is critical to neuronal survival. In the bigenic model, over-expression of α–synuclein also attenuates injury in the CSPα knockout mice, whereas ablation of endogenous mouse α–synuclein exacerbates the toxicity observed (Chandra *et al.*, 2005).

These data, taken with evidence that α–synuclein expression (as determined by mRNA and protein levels) actually diminishes within dying neurons in certain models (Kholodilov *et al.*, 1999a,b) suggest that α–synuclein has a complex and variable role in the neurodegenerative process, and that may differ based on the mechanisms of insult or type of neurotoxicity involved. For example, the studies with paraquat show that increased α–synuclein expression is not always a *de facto* marker of enhanced toxicity or vulnerability, and may even be protective. While this effect may be due to compensation within transgenic models (*i.e.*, increased chaperone expression) and not α–synuclein itself, it is possible that increased levels of the protein may be directly protective to the cell under certain conditions/paradigms, a hypothesis supported by evidence from non-transgenic models (Kholodilov *et al.*, 1999a,b). Clearly further studies are warranted to unravel this apparently complex role of α–synuclein; in particular, determining ways to block its upregulation is currently being explored as a way to slow or halt the progression of PD. Finally, in keeping with the theme of this article, it should be pointed out that these novel findings could only have emerged as the result of the fusing of toxicant and transgenic models (gene-environment modeling), underlining the importance of the approach as a "tool" to ascertain the role of PD-related proteins under normal and disease conditions.

Rotenone and α–*Synuclein:* To date, only a few studies have been carried out with rotenone in transgenic animals. Abnormal PBS- and detergent-soluble aggregates of α–synuclein have been isolated from α–synuclein-overexpressing (as well as wild-type) animals treated with rotenone (Dalfo *et al.*, 2004) and the possibility exists that these forms may represent toxic α–synuclein congeners. Interestingly, however, in at least one line of α–synuclein over-expressing mice neurotoxicity was not enhanced, whereas MPTP neurotoxicity clearly was made worse (Nieto *et al.*, 2006). This observation represents yet another important example of how merging the various models can provide insights that no other approach could, as it suggests that α–synuclein-related effects on MPTP toxicity may be mediated via pathways other than (or in addition to) direct cytotoxicity due to just mitochondrial complex I inhibition.

Parkin-Neurotoxicant Interactions

Although mice deficient in *parkin* do not recapitulate the behavioral or neuropathological features of parkinsonism (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Von Coelln *et al.*, 2004; Perez and Palmiter, 2005), mitochondrial abnormalities and reduced expression of proteins involved in response to oxidative injury have been reported (Palacino *et al.*, 2004), suggesting that parkin deficiency might render mice more susceptible to insult from exposure to exogeneous neurotoxicants. Surprisingly, however, to date there are no reports of parkin deficient mice that have been exposed to MPTP, paraquat, rotenone or LPS. On the other hand, experiments have been carried out in these animals with both 6-OHDA or the terminal-targeting toxin methamphetamine (Perez *et al.*, 2005), but enhanced nigrostriatal injury in parkin null mice was not observed under either of these conditions. In primary midbrain cultures exposed to rotenone, the dose-dependent reduction in the number of TH-positive neurons that occurs is more pronounced in cells obtained from null parkin mice along with increased reactive gliosis (Casarejos *et al.*, 2006). This *in vitro* study suggests that parkin-deficiency may indeed bestow enhanced vulnerability to toxins that target specific pathways, such as those that elicit Complex I inhibition, and that *in vivo* experiments are likely to prove worthwhile.

DJ-1-Toxicant Interactions

Similar to null parkin mice, one would predict that transgenic models of DJ-1 deficiency would be more vulnerable to oxidative injury promoted by exogenous toxicant exposure, particularly because markers of oxidative damage are increased in striatum of aged null DJ-1 mice (Chen *et al.*, 2005). And indeed, as predicted, DJ-1-deficient mice have been reported to be more susceptible to MPTP (and amphetamine-induced nigrostriatal injury) (Kim *et al.*, 2005). However, no alterations have been found in mice exposed to paraquat (Goldberg *et al.*, 2005). This is surprising since paraquat has been shown to elicit the formation of ROS, and therefore would have been predicted to be particularly toxic in the DJ-1 transgenic mouse (Goldberg *et al.*, 2005). One explanation for the failure to see enhanced toxicity may be related to the way that different toxicants gain entry into the nigrostriatal neurons. The pathway utilized for paraquat entry into dopaminergic neurons is not well understood, but both amphetamine and MPP^+ utilize the dopamine transporter (DAT) to access the cytoplasm/terminals of dopaminergic neurons (Javitch *et al.*, 1985). Interestingly, studies in two independent lines of DJ-1 knock-out mice have been shown to exhibit enhanced activity of DAT. Such alterations in the transporter could not only explain the changes in striatal dopaminergic tone that have been reported in these animals, but also their increased vulnerability to injury by increasing the uptake of certain neurotoxicants such as MPP^+, but not others such as paraquat. We are currently investigating these possibilities in our own laboratory as this represents a potential explanation for the differential effects observed among the various neurotoxicants, as well as shedding light on the mechanisms by which DJ-1 deficiency contributes to the demise of nigral dopaminergic neurons in humans.

Part 4. Conclusions

As noted in the introduction, the last quarter century has witness a dramatic increase in the number and variety of models that are currently available to investigate the mechanisms of neurodegeneration in PD. This is good news, as almost nothing is likely to accelerate progress in the field more than having better models to investigate the disease in regard to underlying mechanisms of neuronal degeneration and dysfunction, factors that predispose to the disease, and ways to modify its progression. As described in this article, these models have emerged primarily from two separate disciplines, neurotoxicology and genetics. As useful as these models have proved to be in their own right, it is equally apparent that the models emerging from each of these disciplines have their strengths and weaknesses, and none have been found to fully replicate the features of the disease. What we have tried to capture, and what is both innovative and exciting, is the way these diverse models are increasingly being used in combination with a number of goals in mind. The first is to better emulate the full spectrum of features of the disease, ranging from the widespread α–synuclein-positive neuropathology in the form of Lewy bodies

and Lewy neurites, to the devastating cell loss that is seen in such areas as the substantia nigra and locus ceruleus. The second is to study the effects of genetically and environmentally determined risk factors (gene-environment interaction) in the laboratory, searching for both genetic and environmental determinants that may emulate those that occur in the idiopathic disease. This increasingly popular approach of fusing genetic and toxicant models is in turn creating an entirely new cadre of models that have already yielded some intriguing observations, many of which are outlined in this article, and that would not have been possible using either type of model alone (Table I). This is particularly true when it comes to the study of the underlying mechanisms of neurodegeration in all of these models. Furthermore, fusing these models in a myriad of ways offers the potential of an almost endless array of new experiments. For example, one might consider combining transgenic models over-expressing α–synuclein with the recently described LPS model that induces long-latency toxicity (Qin *et al.*, 2007). Finally, combining these genetic and toxicant models offers an experimental paradigm for unmasking genetically determined susceptibility in transgenic models that have had vexingly little in the way of a parkinsonian phenotype, or conversely, expanding the horizon of toxicant models that are far more selective than the disease itself. Indeed, it may not be too much to hope that there will be many more such models based on the fusion of the transgenic and toxicant paradigms that will increasingly provide researchers with a system that captures the most common events underlying typical PD.

Before closing, it is important to offer a final caveat to all of us who are trying to model the complex disorder we call Parkinson's disease. It is increasingly clear that the time has come to move beyond our decades old and arguably myopic focus on the nigrostriatal system, regardless of what model is being used (Langston, 2006). It is now increasingly apparent the PD is a widespread disease of the central nervous system and the peripheral autonomic nervous system, and that many or

Table I Model Fusion: Effects of Gene-Environment Interactions on Nigrostriatal Toxicity

		SYSTEMIC TOXICANT			
		MPTP	**PQ**	**Rotenone**	**LPS**
GENE	**α-Syn overexpr**	**Enhanced injury** **PrP-A30P;Thy-1-WT;** **TH-A30P+A53T** (Nieto *et al.*, 2006; Song *et al.*, 2004 Richfield *et al.*, 2002) **No difference** **TH-A30P; Thy-1-A30P** (Rathke-Hartlieb *et al.*, 2001)	**Neuroprotection** **TH-A53T;TH-WT** (Manning-Boğ *et al.*, 2003)	**No difference** **PrP-A30P** (Nieto *et al.*, 2006)	**n.d.**
	α-Syn deficient	**Neuroprotection** (Dauer *et al.*, 2006; Schlueter *et al.*, 2003; Drolet *et al.*, 2004; Klivenyi *et al.*, 2006)	**n.d.**	**n.d.**	**n.d.**
	DJ-1 deficient	**Enhanced injury** (Kim *et al.*, 2005)	**No difference** (Goldberg *et al.*, 2005)	**n.d.**	**n.d.**
	Parkin deficient	**n.d.**	**n.d.**	**n.d.**	**n.d.**

n.d. = not determined; this matrix represents experimental evaluation of gene-toxicant interactions in existing PD mouse models. The studies highlighted in this table reported on markers of frank nigrostriatal toxicity such as nigral dopaminergic cell death and striatal dopamine and metabolite levels. Fusing of genetic and toxicant models offers an entire array of new experiments (as noted by n.d.) for evaluation, will expand even further with the development of new transgenic mice and toxicant paradigms.

most of these areas of anatomical involvement are producing signs and symptoms that plague patients affected by the disease (Langston, 2006). Yet, with notable exceptions, only cursory non-motor behavioral assays and anatomic surveys are typically carried out in these models, which may result in failure to capture other significant features of the disease, some of which may far precede the degeneration of the substantia nigra by many years in patients with PD. In the future, much broader surveys of behavior and neuroanatomical features of these models should be assessed if we are to better understand and treat all aspect of the disorder and better care for the patients who are its victims.

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