SYSTEMIC BACTERIAL ENDOTOXIN PLUS MPTP AS A MODEL OF PARKINSON'S DISEASE IN C57BL/J6 MICE

by

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Introduction

Historically, animal models of Parkinson's disease (PD) have focused on mimicking end-stage pathology by inducing degeneration of substantia nigra, pars compacta (SNc) neurons and the resultant loss of dopamine (DA) in the striatum. Although these models have been successful in accomplishing this main goal (through a variety of means), they have largely failed to make a significant contribution towards the development of new treatments and tools for early diagnosis. One reason for this shortcoming is that the relatively slow progression of DA depletion believed to occur in the sporadic or idiopathic form of PD has not been effectively reproduced in animals. Moreover, efforts made toward integrating theories of PD etiology into animal models have been only moderately successful. This lack of progress may in part be due to a tendency for past research to focus on the etiology of PD as being related to a single factor. More recent theories of PD etiology have suggested that multiple factors interact synergistically and may be responsible for disease onset and progression. The present study attempted to build upon recent theories and *in vitro* research to develop an *in vivo*, systemically-administered mouse model of PD by focusing on mimicking the progression of PD with two factors, each known to trigger some parkinsonian symptoms. The two factors chosen were the bacterial endotoxin, lipopolysaccharide (LPS) and the neurotoxin, *1*-methyl-*4*-phenyl-*1,2,3,6*-tetrahydropyridine (MPTP). These factors test the possibility that inflammation in the brain (LPS) and subsequent exposure to exogenous toxins (MPTP) are one cause of PD.

Neuropathology of PD

PD is the second most common neurodegenerative disorder in the United States (Pfeiffer 2005), but its cause is unknown. The neuropathology of PD is unique; the most noted pathology is loss of dopaminergic neurons located in the SNc. SNc degeneration is most prominent in the ventral cell groups, which project to the putamen portion of the striatum (Hornykiewicz, 1998). These SNc neurons also project to the caudate nucleus, the globus pallidus, the olfactory tubercle, and ascend into cortical areas, including the prefrontal cortex (Burns, 1991; Haines, 2004). Furthermore, SNc degeneration appears to be gradual and progressive and the degree of neuronal loss has been shown in postmortem examination to be related to the duration of disease (Hirsch et al., 2003). The net effect of loss of SNc dopaminergic neurons is a subsequent decrease of DA in both the SNc and the striatum. For instance, even in early, untreated PD, patients show striatal DA uptake that is bilaterally reduced to 39 percent of controls (Haapaniemi et al., 2001). The consequence of this DA reduction is altered physiological responses from the SNc. First, surviving DA neurons increase output and striatal DA receptors compensate for reduced DA by increasing numbers of postsynaptic receptor sites (Hornykiewicz, 1998). Second, decreased striatal DA causes a downstream excess of neuronal activity in the subthalamic nucleus and internal segment of the globus pallidus (Fahn, 2003) and behaviorally, the consequence of decreased striatal DA is generalized motor dysfunction. Furthermore, decreased striatal DA also causes reductions in the major metabolites of DA, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) (Hornykiewicz, 1998).

There are additional neuropathologies unique to PD. In surviving SNc dopaminergic neurons, intracytoplasmic protein inclusions called Lewy bodies are found (e.g., Orth & Tabrizi, 2003). Lewy bodies are deleterious to neurons and in PD, are comprised of two major proteins, alpha synuclein and ubiquitin, both of which are believed to be somehow involved in the pathogenesis of the disease (Goldberg & Lansbury, 2000). Furthermore, in addition to the SNc, postmortem examination of human PD brains has shown extensive extrastriatal and nigral neurodegeneration. Lewy bodies are also typically seen in the dorsal glossopharyngeal and vagal nuclei, the olfactory bulb, the anterior olfactory nuclei, neurons of the intermediate reticular zone, the serotonergic neurons of the locus coerulus and raphe nuclei, and many subnuclei of the thalamus and amygdala (Braak et al., 2003a; Del Tredici et al., 2002; Hawkes, Shephard, & Daniel, 1997).

Symptomology of PD

Idiopathic PD is identified by the presence of certain so-called cardinal behaviors: bradykinesia (slowness of movement), a tremor of the distal musculature at rest (usually the hands), axial musculature rigidity, and postural instability (Lindvall & Björklund, 2004; Pfeiffer, 2005). PD can also be further diagnosed based on a patient's responsiveness to the standard pharmacological treatment levodopa and by the degree of gait impairment (Fahn, 2003; Pfeiffer, 2005). There are additional symptoms often seen in PD that are considered to be secondary to the cardinal features. These include micrographia (handwriting abnormalities, usually writing of diminished size), reduced facial expression ("masked face"), reduced arm swing when walking, gastrointestinal dysfunction, and insomnia (Fahn, 2003; Pfeiffer, 2005). Furthermore, these symptoms do not typically emerge until approximately 80 percent of SNc neurons have disappeared, perhaps due to failure of

previously-intact, compensation from remaining DA neurons (Hornykiewicz, 1998). Finally, as the SNc neurodegeneration progresses, parkinsonian symptoms continue to worsen.

Etiology of PD

Although the discovery of an actual cause of PD remains elusive, there are many prominent theories regarding its etiology including (but not limited to): exposure to environmental neurotoxins, heavy metals, genetics, and factors related to lifestyle such as rural living (Fukuda, 2001; Tanner, 2003). In fact, it is now thought that it is likely a heterogenous syndrome that has more than one causal factor (Lang & Obeso, 2007; Orth & Tabrizi, 2003; Pfeiffer, 2005). Therefore, development of idiopathic PD may be the end result of the interactions between a variety of factors such as those relating to the individual (e.g., genetics), the innate vulnerability of the SNc (e.g., immunological sensitivity) and/or to the external environment (Liu, Gao, & Hong, 2003a).

A number of environmental toxins have been correlated with parkinsonism, including pesticides, certain metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide. However, to-date, no specific environmental toxin has been reported in the brains of PD patients (Olanow & Tatton, 1999).The most compelling evidence for an environmental factor in theories of etiology in PD stem from studies on *1*-methy-*4*-phenyl-*1,2,3,6*-tetrahydropyridine or MPTP, a substance known for its ability to cause a form of parkinsonism. MPTP is a potent neurotoxin which was discovered in the early 1980s when a geographically and temporally isolated outbreak of a parkinsonian syndrome led doctors to search for a toxic cause (Langston et al., 1983). The culprit was identified as a batch of synthetic heroin; it was believed that the illicit chemist who created it must have been trying to make *1*-methyl-*4*-pheny-*l-4*-propionoxypiperidine (MPPP), an analogue of an opiate called

meperidine (Ballard, Tetrud, & Langston, 1985). MPTP is similar to meperidine in chemical structure and since some striatal opiate receptors are located on dopaminergic terminals, opiates or compounds with opiate-receptor affinity may modulate the activity of dopaminergic neurons (Diamond & Borison, 1978). Interestingly, the neurodegenerative effects and behavioral symptoms of MPTP were so remarkably similar to idiopathic PD that it led researchers to believe that if a naturally-occuring analogue of MPTP could be identified in the environment, then it could very well be the cause of PD itself.

There are multiple, parallel mechanisms that mediate MPTP neurotoxicity. MPTP binds centrally with high affinity to monoamine oxidase B, which transforms MPTP into its toxic metabolite *1*-methyl-*4*-phenylpyrindium (MPP^+), which then binds with high affinity to the dopamine transporter whereby the compound gains access to presynaptic DA terminals (Snyder & D'Amato, 1986). One mechanism by which MPTP exerts its neurotoxic action is by inhibition of complex I of the mitochondrial respiratory chain. This blocks electron transport and depletes adenosine triphosphate (ATP), the main cellular energy source (Fukuda, 2001; Przedborski et al., 2004). A consequence of severe energy impairment is decreased activity of the energy-dependent calcium-ATPase which leads to excitotoxicity via intraneuronal calcium overload. Elevated intracellular CA levels activate degradative enzymes like phosphatases and proteases, which will ultimately lead to excitotoxic cell death (Schmidt & Ferger, 2001). Second are those processes which are in response to the initial neuronal insult, which include elements of the apoptotic pathways such as Bax; the JNK pathway, and caspases (Eberhardt & Schulz, 2003; Nicotra & Parvez, 2002; Przedborski & Vila, 2003). Additional mechanisms amplify the neurodegenerative insult, which include induction of various proinflammatory factors such as prostaglandin and the production of

superoxide which causes oxidative stress (Przedborski et al., 2001; Whitton, 2007). In fact, much of the current theoretical knowledge regarding possible neurodegenerative mechanisms in PD stem from research with MPTP and indeed, this compound has afforded researchers many important insights (Dunnett & Björklund, 1999). However, since MPTP patients examined showed no evidence of Lewy bodies (Langston et al., 1999), an important pathological hallmark of the disease, it seemed other factors may also be involved in idiopathic PD development.

More recently, theories of PD etiology which implicate inflammatory processes have received much attention. In general, inflammation is a beneficial response in that it is a defensive reaction that eliminates or neutralizes harmful stimuli, which ultimately aids in the maintenance of tissue integrity (Minghetti, 2005). Inflammation in the brain involves glial cells, namely microglia and astrocytes. Although astrocytes are important, microglia are the immune cells thought to be the most actively involved in the neurodegenerative process (Block & Hong, 2005): In an adult, microglia comprise approximately 12 to 20 percent of the brain and come in two forms: resting or activated. Resting microglia exhibit a characteristic ramified morphology and perform immune surveillance; they are the resident immunocompetent and phagocytic CNS cells and for the majority of the time, their activity goes unnoticed (Block & Hong, 2005; Whitton, 2007). However, resting microglia seem to be highly sensitive to changes in their microenvironment and therefore, in response to injury or other stimuli, the morphological state of microglia changes to amoeboid activation (Block & Hong, 2005; Liu et al., 2003a; Whitton, 2007). Although activated microglia are known to be neuroprotective in some instances (i.e., release of trophic factors) (Morgan et al., 2004; Polazzi et al., 2001), most of what is released is proinflammatory (e.g., tumor necrosis factor

alpha; TNFα) and/or causes oxidative stress (Liu et al., 2003a; Minghetti, 2005; Whitton, 2007). It is known that microglia activation is an early event in Alzheimer's disease and is implicated in its pathogenesis (Chong et al., 2005). Also, microglia become activated following head injury and are implicated in a form of PD referred to as dementia pugilistica, a type of parkinsonism typically seen in boxers (Plassman et al., 2006).

Accumulating evidence implicates inflammation in idiopathic PD as well. In a seminal study, McGeer, Itagaki, and McGeer (1988) reported large numbers of human leukocyte antigen-positive reactive microglia in the SNc of PD brains, as compared to normal controls. Also, in postmortem analysis, brains of three MPTP users were analyzed. Even though exposure to MPTP was brief and many years prior, neuropathology was highly similar to that of an idiopathic PD brain and clusters of activated microglia were found surrounding many surviving SNc neurons (Langston et al., 1999). It is not known whether the presence of these activated microglia was a cause of, concurrent with, or result of SNc degeneration. Nevertheless, it is now the case that inflammation is no longer thought to be merely epiphenomenal to the neurodegenerative process; rather, it is more likely an active contributor (Block & Hong, 2005; Casals, Elizan, & Yahr, 1998; Hirsch et al., 2003; Jellinger, 2004; Liu et al., 2003a; Plassman et al., 2006; Teismann et al., 2003; Whitton, 2007). Experimental research offers support to this notion. Use of 6-hydroxydopamine (6-OHDA), a catecholaminergic neurotoxin, in PD rodent models found that chronic, toxin-induced cell death is not, in and of itself, enough to induce the expression of inflammatory markers (proinflammatory cytokines in this case) to the level of that seen in idiopathic PD patients. It was concluded that an additional stimulus is needed in the PD animal model to accurately reproduce such pathology (Depino et al., 2003).

Another interesting aspect about the role of inflammation as causal in PD is that the SNc seems to be a structure that is itself particularly vulnerable to inflammatory activity. It will sooner succumb to the damaging effects of activated microglia and other, potentially detrimental processes such as oxidative stress. For instance, recent evidence has shown that the SNc has the highest population of resident microglia in the brain, somewhere between three and five times more than other regions (Herrera et al., 2000; Kim et al., 2000). Additional recent data also suggested that this subset of microglia may be actively deleterious to dopaminergic neurons in PD, in particular, for those SNc dopaminergic neurons that are damaged or already dying (Banati, Daniel, & Blunt, 1998). Furthermore, the highly oxidative conditions that are present in dopaminergic neurons (related to their high neuromelanin content) have led to speculations that oxidative stress may be an important factor in the neurodegeneration seen in PD (Whitton, 2007). Psychological stress may also play a part in the pathogenesis of PD; it can incite inflammation and proinflammatory cytokine activation and is associated with various phenomena, both peripherally and centrally (e.g., various immunologic, neurochemical, neuroendocrine, and behavioral effects) (Kronfol & Remick, 2000). Psychological stress also increases the central expression of factors that have the capacity to be neurotoxic (e.g., proinflammatory cytokines) (Smith, Castro, & Zigmond, 2002).

Therefore, SNc microglia may respond to dying SNc dopaminergic neurons by releasing neurotoxic inflammatory factors, a cycle termed reactive microgliosis, thus speeding the demise of these neurons (Block & Hong, 2005; Lang, 2007; Teismann et al., 2003). As reported in Langston et al. (1999), in the cases of the MPTP addicts where the cause of the degeneration was known, a PD diagnosis was still made years after the insult

responsible for the neurodegeneration was present. The data of Langston et al. (1999) suggest that microglia activation started at the time of exposure and was possibly amplified through the reactive microgliosis process (Block & Hong, 2005). Indeed, it is now known that the microglial response to injury or damage in the CNS is long and self-perpetuating (Gao et al., 2003a,b; McGeer et al., 2003). However, parkinsonian degeneration is not that simple. It remains unknown exactly what mechanisms govern the observed degeneration in idiopathic PD and other parkinsonian states (e.g., MPTP-induced parkinsonism) and mounting evidence suggests that acute microglial activation alone is not enough to lead to long-term SNc cell death (e.g., Iravani et al., 2005). Therefore, efforts to link the etiology of PD to a single, causative factor remain insufficient.

Animal Models of Parkinson's Disease

Animal models are critical to aid in our understanding of human diseases states and they provide invaluable opportunities to evaluate new therapies (Jakowec & Petzinger, 2004). For the most part, it is important for a model to resemble as closely as possible the neurochemical, neuropathologic, and behavioral features of the human condition. Other considerations that may enhance the value of a model include cost, time required or efficiency, reproducibility, and animal mortality (Jakowec & Petzinger, 2004). In PD research, animal models are varied in their routes of dopaminergic degeneration; yet despite the varied methods of induction of parkinsonian pathology, the ultimate aim of all animal models of PD has been to reproduce the relatively specific nigrostriatal dopaminergic cell death and decrease of striatal DA that is seen in the end-stages of the idiopathic form of the disorder (please refer to Table 1). Furthermore, as idiopathic PD appears to be rather heterogenous in origin, these models are helpful in gaining a better understanding of the

multiple biochemical and molecular pathways that lead to the clinical PD phenotype (Orth & Tabrizi, 2003).

Table 1. *Summary of the Major Animal Models of Parkinson's Disease.*

MODEL	Advantages	Disadvantages
6-OHDA	Nigrostriatal DA loss; well-documented behavior; pharmacology	Instaneous depletion; unilateral lesions; no LBs
MPTP	Nigrostriatal DA loss, parkinsonism in humans; simple administration method	Neurochemical recovery; no LBs, inconsistent behavioral and neurochemical effects
Rotenone	Nigrostriatal DA loss; LBs; some parkinsonian behavior	Variable pathology; mortality with high doses
Genetic (e.g., alpha synculein)	Allow for investigation of specific proteins in PD; etiological link for small percentage of cases	Typically no nigrostriatal DA loss or parkinsonian behaviors
Bacterial endotoxin (e.g., LPS)	Nigrostriatal DA loss; relatively slow and progressive;	Inflammation seen in spectrum of neurodegenerative disease; parkinsonian behavior not reported

Note. *6-OHDA* – 6-hydroxydopamine; *DA* – dopamine; *LBs* – Lewy bodies; *MPTP* – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *SNc* – substantia nigra, pars compacta; *PD* – Parkinson's disease.

The 6-OHDA Model of PD

Along with MPTP, the catecholaminergic neurotoxin 6-OHDA is considered to be the most commonly-used and well-characterized rodent model of PD (Jenner, 2002; Schober, 2004). Rats injected unilaterally into the ascending dopaminergic pathway with 6-OHDA show motor patterns that mirror many of the sensorimotor abnormalities seen in PD patients including tremor (Salamone et al., 1998), decreased stride length (Schallert et al., 1978), and postural rigidity (Schallert et al., 1979; Schallert & Tillerson, 2000). Neurochemically, this model produces an acute depletion: high doses of 6-OHDA produce an almost instantaneous

and complete ablation of neurons in the nigrostriatal DA system, with upwards of 97% DA depletion (Cenci & Lundblad, 2005; Ungerstedt, 1968). The advantages of the 6-OHDA model include the availability of a number of quantitative behavioral tests that are sensitive to 6-OHDA-induced nigrostriatal damage and since 6-OHDA lesions are normally unilateral, asymmetrical behavior allows for each subject to serve as a within-animal control. Interestingly, endogenous 6-OHDA has been reported in elevated amounts in the urine of L-dopa-treated PD patients and in the caudate of normals (Andrew et al., 1993). Incidentally, L-dopa treatment itself is thought to hasten DA terminal loss (Fahn et al., 2004). Yet unlike the MPTP addicts, no known cases of 6-OHDA-induced parkinsonism exist in humans.

There are some disadvantages of the 6-OHDA model of PD. These include the fact that bilateral lesions render animals anorectic and unable to care for themselves. Also, administration of the toxin requires costly and time-consuming surgical procedures because systemically administered 6-OHDA does not cross the blood-brain barrier (Schober, 2004). It has been noted that the rotational behavior often exhibited by 6-OHDA-injected animals is an artificial behavior with no certain counterpart in human PD patients (Ungerstedt, 1976). Also, with the possible exception of chronic dosing regimens (e.g., Fornai et al., 2005), in the 6-OHDA model, formation of Lewy bodies is not typically seen (Cenci, Whishaw, & Schallert, 2002; Orth & Tabrizi, 2003). Finally, the rapid nature of the neuronal destruction does not mimic the slow and progressive nature of the human form of PD. Nevertheless, 6-OHDA remains an important tool for certain aspects of PD research, such as the assessment of secondary damage following complete removal of DA input into the striatum (Cannon et al., 2005) and end-stage PD treatment strategies (Cenci & Lunblad, 2005).

Other PD Models

Additional classes of models are those that utilize pesticides such as rotenone and paraquat, and those models that involve the manipulation of a gene that codes for a protein known to be involved in the pathogenesis the disease, e.g., alpha synuclein. Pesticides were identified as possible dopaminergic neurotoxins because of their structural similarities to MPTP (Grünblatt, Mandel, & Youdim, 2000; Liu et al., 2003a; Rajput & Uitti, 1987). Pesticide models can reproduce many of the pathological and behavioral characteristics of PD including motor dysfunction, nigrostriatal DA depletion, microglia activation, and formation of protein inclusions in SNc neurons (Gao et al., 2003b; Alam & Schmidt, 2002). However, there is a reported high variability in responsiveness to pesticide administration, with sometimes only half of animals showing development of nigral lesions (Betarbet et al., 2000). Also, experimental models that employ pesticides use relatively very high doses in comparison with a normal human population exposure level and more importantly, even though traces of some pesticides have been found in the brains of PD patients, these population data are merely correlational at present (Liu et al., 2003a).

Models of PD have been developed for research using genetic knockouts (KO) or transgenic (TG) animals. KO models involve the mouse genome being manipulated to remove a DNA sequence so a gene cannot function (Fleming, Fernagut, & Chesselet, 2005; Orth & Tabrizi, 2003). KO mice model recessive disorders where the phenotype results from the loss of the dysfunctional gene. One genetic model researchers use in PD is an alpha synuclein KO and recently, a dopamine transporter (DAT) KO has also been evaluated (Fleming et al., 2004; Tillerson, et al., 2002a). Both models show increased resistance to the neurotoxic effects of MPTP and MPP^+ and some locomotor impairment. Alpha synuclein KO

mice also show some locomotor impairment at one-year of age, however, they lack nigral dopaminergic cell loss, no fibrils (a key morphological feature of Lewy bodies) and pathology changes involving protein inclusions in the spinal cord (not seen in PD) (Sommer et al., 2000; van der Putten et al., 2000). TG models express a gene of interest to the host organism (Orth & Tabrizi, 2003). Most TG models in PD have focused on alpha synuclein (Sommer et al., 2000). These mice show intracytoplasmic protein inclusions in the neocortex, hippocampus, olfactory bulb, and the SNc. Advantages of genetic models is that they allow for a more complete understanding of the function of the protein under manipulation and its role in disease and as a therapeutic target (e.g., Lang & Obeso, 2004). Disadvantages of genetic models include that the animals only sometimes develop parkinsonism (Fleming et al., 2004) and pathology can sometimes extend into areas not affected in idiopathic PD (Fleming, Fernagut, & Chesselet, 2005).

The MPTP Mouse Model of PD

Since its accidental discovery in the 1980s, MPTP has become one of the most commonly used agents in animal models of PD. MPTP has an advantage over other toxic models because it is known to induce a syndrome in humans virtually identical to PD (Przedborski & Vila, 2003). It has been commonly administered in various doses and routes in non-human primates and mice. Generally, in the C57BL/6J mouse strain, MPTP induces striatal DA content loss (e.g., Bradbury et al., 1986; Heikkila et al., 1984; Sedelis et al., 2000). However, comparisons of DA loss across studies has proven to be rather problematic since MPTP dosing regimens vary greatly: single injection doses range from as low as 4 milligrams to as high as 50 milligrams with cumulative doses varying even more (Hoskins & Davis, 1989; Jakowec et al., 2004; Kohutnicka et al., 1998; Petzinger et al., 2007; Sonsalla &

Heikkila, 1986; Willis & Donnan, 1987). In some studies in the C57BL/6J mouse, MPTP has been reported to produce an almost complete, permanent, and selective nigrostriatal DA depletion similar to that seen in humans and primates (Jakowec et al., 2004; Sundström et al., 1987). DA levels in the striata of MPTP-treated mice in the time period immediately after treatment show a steady decline as dose increases (Haobam et al., 2005; Sundström, Fredriksson, & Archer, 1990). However, much of the neurochemical data to-date in the MPTP mouse model of PD also indicate that in the C57BL/6J strain, MPTP causes a depletion of striatal DA without causing nigral cell loss or any permanent neurochemical damage (Jackson-Lewis et al., 1995; Jakowec et al., 2004; Willis & Donnan, 1987). Furthermore, a recurring finding reported in MPTP use is that striatal DA often recovers to near-normal levels (Arai et al., 1990; Jakowec et al., 2004; Ricaurte et al., 1986; Tillerson et al., 2002b) and although MPTP temporarily reduces striatal DA content, dopaminergic neurons in the SNc often remain structurally undamaged (Arai et al., 1990). Furthermore, the administration paradigm, i..e., number of injections, dose administered, duration of analysis, etc., profoundly affects the amount of dopaminergic damage (Bezard et al., 1997; Schober, 2004; Schmidt & Ferger, 2001). Thus the ability of MPTP to induce a complete pathological picture of PD remains inconclusive (Hardy & Lees, 2005).

Behavioral Effects of MPTP Administration in Mice

Although excellent tests exist for evaluating motor behavior in rats, abnormalities of motor behavior are more challenging to detect in mice, particularly in bilateral-pathology models (Fleming et al., 2004). In MPTP research, behavioral results vary widely, with some reporting no behavioral deficits (Willis & Donnan, 1987), behavioral deficits with recovery (Jakowec et al., 2004; Rozas et al., 1998), or apparently permanent behavioral effects

(Petroske et al., 2001). Generally, when behavioral abnormalities are reported to exist, they are typically transient and parkinsonian in nature such as akinesia (or bradykinesia), muscle rigidity, and gait and postural disturbances (Sedelis, Schwarting, & Huston, 2001). Other reported short-term behavioral effects of hypersalivation, piloerection, seizures, and hypokinesia (Earl & Sautter, 2002; Schmidt & Ferger, 2001). However, as previously mentioned, the MPTP dosing regimens used vary widely and thus, behavioral data often appear contradictory (Liu et al., 2003b; Fornai et al., 2005; Mori et al., 2005; Sonsalla & Heikkila, 1986; Willis & Donnan, 1987). As with neurochemical data, this variation in MPTP regimens renders comparison of behavioral effects across studies problematic.

Shortened stride length is one of the chief, abnormal characteristics of gait in PD patients (Amende et al., 2005; Fernagut et al., 2002; Nieuwboer et al., 2001). Such behavior is thought to be mediated by the basal ganglia, which play an important role in the regulation and maintenance of gait and movement (Hausdorff et al., 1998) and in PD, these nuclei function abnormally due to the ongoing dopaminergic degeneration. Mice show a consistent and quantifiable gait that allows for the analysis of gait characteristics into the evaluation of PD models (Clarke & Still, 1999). In one study, mice receiving MPTP were found to exhibit reduced stride length over control animals, and the degree of stride length impairment was related to the degree of SNc neuronal degeneration (Fernagut et al., 2002). Another group used C57BL/6J mice to analyze movement on a transparent treadmill following three days of once daily injections of 30 mg/kg MPTP (90 mg/kg total). Mice were videotaped and images were analyzed using applied ventral plane videography, which generates digital paw prints that are used in an analysis of gait (Amende et al., 2005). It was found that MPTP mice had significantly shorter stride length and increased stride frequency compared to controls. Such

a gait disturbance reflects those widely reported in human PD patients (Nieuwboer et al., 2001).

Others report behavioral deficits in tests of locomotion. C57BL/6J mice showed significantly decreased spontaneous locomotor activity in an open-field paradigm and it was concluded that a short, high-dose MPTP regimen can lead to marked, long-term deficits in locomotor activity (Sundström et al., 1990). Other research with mice given MPTP in the open field showed reduced total mean distance traveled and distance decreased as dose (40, 60, & 80 mg/kg) of MPTP increased (West et al., 2006). Additionally, a chronic regimen of MPTP (25 mg/kg, twice weekly) given over 5 weeks was also found to produce long-term motor deficits in the rotarod test (Petroske et al., 2001). Others reported a high-dose of MPTP initially produced rotarod deficits that recovered over time (Rozas et al., 1998). Therefore, in the MPTP mouse model, it may be that detection of behavioral abnormalities is highly dependent upon the MPTP dosing regimens and sensitivity of the behavioral measures, thus the variation across studies in experimental design may be responsible for the spectrum of often contradictory results.

The Bacterial Endotoxin Model of PD

Since McGeer et al. (1988) reported activated microglia in the SNc of PD brains, inflammatory processes have been investigated as possible mechanisms of PD neuropathology. One way to induce a nonspecific inflammatory response is with what is considered to be the prototypical bacterial endotoxin, lipopolysaccharide (LPS). LPS is an immunogen derived from cell wall of Gram-negative bacteria. LPS mediates many of the host-pathogen infection properties without actually infecting the organism (e.g., Whitton, 2007). Systemic LPS administration induces an inflammatory response in peripheral nervous

system that results in the production of proinflammatory cytokines (e.g., IL-1β, IL-6, TNFα), which can activate central inflammatory processes (Kronfol & Remick, 2000; Perry, 2004). Prenatal exposure to LPS, used as a model of a complication of pregnancy called bacterial vaginosis, was shown to increase adulthood DA neuron vulnerability in that as animals aged, nigral DA cell loss increased (Carvey et al., 2003). Furthermore, these animals exhibited reduced striatal DA as well as increased DA activity (HVA/DA) and elevated levels of TNFα throughout the duration of their lives (16 months), results concurrent with clinical PD literature (Carvey et al., 2003). It was concluded that the increases in reactive oxygen species and TNFα in conjunction with increased DA turnover create a self-perpetuating toxic cycle, which may be largely responsible for the progressive nature of the neurodegeneration in PD (Carvey et al., 2003). Also, after repeated LPS administration, cytokine expression in the CNS can still occur even when the system is exhibiting peripheral tolerance (Chen et al., 2005).

Further studies have suggested that single, intranigral-infusion and chronic LPS-exposure is selective in destroying only the dopaminergic neurons in the SNc of rats (Castaño et al., 1998; Iravani et al., 2005). *In vitro* work shows dopaminergic degeneration and also suggested peak DA loss may be delayed up to six weeks after cessation of LPS administration (Gao et al., 2002b; Le et al., 2001). Furthermore, these LPS models are some of the first to mimic the slow and progressive nature of SNc cell death that is thought to occur in PD (Block & Hong, 2005).

The apparent involvement of inflammation in a variety of neurodegenerative diseases implies that inflammation may predispose the brain to progressive cell death (McGeer et al., 1988). Nonetheless, inflammation is a general process that affects the entire brain, not only

the dopaminergic system. An important question that remains is how does this inflammation-induced predisposition become "focused" in a way that leads to Alzheimer's disease (for example) in one patient and PD in yet another? It seems reasonable to surmise that there must be other factors that affect the brain in addition to inflammation that cause the neurodegenerative process to be directed in unique ways consistent with the known spectrum of disorders. Thus, in true cases of PD, we should look at factors that specifically lead to cell death in the SNc.

Although it is tempting to hypothesize that inflammation is the initial event that predisposes the brain to progressive degeneration, this need not be the case. Despite the undeniable presence of inflammatory markers in PD, it holds true that much of the clinical analysis is performed in populations that are in advanced stages of disease. Therefore, the question of whether the inflammation seen is primary, concurrent, and/or secondary to the neurodegenerative process and whether neuroinflammation is beneficial or harmful to neurons remains unanswered (Liu et al., 2003a; Hirsch et al., 2003).

Mutlifactor Factor Models: LPS and MPTP

Collectively, research utilizing various PD models has afforded much insight into the disorder. Nevertheless, the cause of idiopathic PD remains unknown as does the availability of effective, long-term treatment (as alternatives to L-dopa; e.g., stem/fetal cell therapy; Lindvall & Björklund, 2004) that may prevent degeneration of SNc neurons and even uncover the etiological mechanisms that may initiate PD neurodegeneration and pathology (Dawson, Mandir, & Lee, 2002; DiMonte, 2003; Earl & Sautter, 2002; Lang & Obeso, 2004).

Surprisingly, there have been only a relatively small number of studies conducted that stemmed from etiological theories that the causes of PD are likely multifactorial (Gaoet al., 2003a). In an *in vitro* analysis of primary mesencephalic cultures, a synergistic neurotoxicity between LPS and MPTP was shown. The synergistic toxicity was more prominent when exposure was for a longer period (7 versus 3 days) and to low doses of each agent. However, when LPS or MPTP were administered alone, neither was more than minimally toxic to neuron-glia cultures (Gao et al., 2003a). Furthermore, in the absence of microglia, the synergistic toxicity was not seen suggesting that inflammatory factors produced by microglia are a necessary part of the toxic cascade. Another study used MPP^+, the toxic metabolite of MPTP, and infused it into the medial forebrain bundle of rats pretreated with a single intraventricular injection of LPS 48 hours prior (Goralski & Renton, 2004). Two days following MPP^+ infusion, both saline- and LPS-pretreated rats showed a 90 percent decrease in striatal DA. Seven days later, saline-pretreated animals recovered to normal DA levels but the LPS-pretreated group showed continued reductions of striatal DA (Goralski & Renton, 2004). Further research confirms that specific factors in the proinflammatory cascade play an important role in the toxicity of MPTP administration. When proinflammatory components of microglia activation such as superoxide (Wu et al., 2003), prostaglandins (Feng et al., 2002; Teismann et al., 2003 a,b), COX-2 (Vijitruth et al., 2006) and TNFα (Sriram et al., 2002) are inhibited, the neurodegenerative effects of MPTP are significantly reduced. Importantly, these studies highlight that inflammatory factors and MPTP exposure can act together to amplify the neurodegenerative process. However, these data remain unconfirmed in an *in vivo* model that is exposed to both agents (i.e., endotoxin and MPTP) systemically and furthermore, the behavioral effects of such treatment are unknown.

The Present Study

It is known that MPTP may reproduce a parkinsonian syndrome in mice (e.g,. Sundström et al., 1990), although it is often reported behavioral and neurochemical recovery occurs after MPTP treatment (Arai et al., 1990; Jakowec et al., 2004; Ricaurte et al., 1986; Tillerson et al., 2002b). It has been hypothesized that the addition of compounds to the MPTP mouse model would enhance MPTP toxicity and allow parkinsonism to emerge consistently (Schmidt & Ferger, 2001). LPS, through activation of microglia, has also been shown to be toxic to SNc neurons (Carvey et al., 2003; Castaño et al., 1998; Iravani et al., 2005; Le et al., 2001). However, because it is also present in other neurodegenerative diseases, inflammation alone cannot account for the full spectrum of pathology seen in PD and the determination of whether it plays a role in disease progression or is a consequence of ongoing neurodegeneration remains unclear (Perry, 2004). It is also known that microglia-mediated inflammatory processes are important in the toxic effects of MPTP (Wu et al., 2002). Furthermore, it is known that *in vitro*, low concentrations of LPS and MPTP together may be selectively and synergistically neurotoxic to dopaminergic neurons, more so than either agent alone (Gao et al., 2003a). To this end, the present study intended to examine the behavioral and neurochemical effects of a low-dose, systemic exposure to the neurotoxin MPTP after LPS-induced systemic inflammation as potential, environmental model of PD.

Method

Subjects

Subjects were 161 experimentally-naïve male C57BL/6J mice (Texas Christian University breeding colony), all approximately nine weeks of age at the start of behavioral testing. At weaning age (30 days), mice were separated from littermates and placed with other male weanlings in group housing. Housing comprised a Plexiglas cage with corncob bedding. Individual cages of 3–4 mice were randomly assigned to treatment groups. Treatment conditions within a cage were kept the same to prevent cross-contamination between animals receiving MPTP to those that did not. All animals had *ad libitum* access to food and water and were maintained on a 12/12 light/dark cycle (lights on at 7am). Animals remained thus for up to four months, at which time the behavioral testing was completed and animals were sacrificed for neurochemical analyses. All procedures were approved by the TCU Institutional Animal Care and Use Committee (IACUC).

LPS/MPTP Administration

One week prior to the commencement of LPS administration, mice were pretested in both behavioral measures to ensure adequate performance ability and to obtain pre-injection data. Next, animals were randomly assigned to one of four conditions: (i) saline, (ii) LPS+MPTP, (iii) MPTP-only, or (iv) LPS-only. Injections were administered as follows: mice received three intraperitoneal (i.p.) injections of LPS (*Escherichia coli* serotype 0111:B4; 250μg/kg in 0.9% sterile saline; Sigma, St Louis, MO) once daily for three consecutive days to achieve significantly elevated central proinflammatory cytokine expression (e.g., Kohman et al., 2007a) or saline. Four hours following the final LPS injection, mice received one low-dose, subcutaneous injection of MPTP-HCl (15 mg/kg;

Sigma, St. Louis, MO) dissolved in 0.9% sterile saline or saline, administered via a 31 gauge, 300µL insulin syringe (BD Pharmingen, New Jersey, USA). The present injection paradigm was timed to achieve maximum systemic toxicity with minimal toxin exposure. Furthermore, an additional cohort of mice received an extra day of injections, i.e., four days of LPS plus two MPTP injections, four days of LPS-only, or two days of a single MPTP injection, making seven groups in total. Since there were no significant differences in any measures within a treatment condition (LPS-only, MPTP-only, or LPS+MPTP), groups receiving the same treatment were combined for all analyses. Finally, all injections were administered during the animals' light cycle.

Behavioral Testing

Behavioral testing consisted of two tests: open field and stride length analysis. Pretesting began one week prior to LPS/MPTP treatment and then continued incrementally from post-injection days one through four, then weeks one through four, and finally every two weeks until week 16 (or approximately the four-month mark). There were 13 total testing sessions with two days of testing per session, i.e., one behavioral test per day. Order of behavioral testing was randomized; for instance, on week eight of testing a group might have been tested in open field on Tuesday and then in stride length on Thursday. All behavioral testing began approximately two hours after the start of the animals' light cycle.

Stride Length Analysis

Shortened stride length is one of the chief, abnormal characteristics of gait in PD patients (Amende et al., 2005; Fernagut et al., 2002; Nieuwboer et al., 2001). Mice show a consistent and quantifiable gait that allows for the analysis of gait characteristics in the evaluation of PD models (Clarke & Still, 1999). To measure stride length, animals were

trained to walk through a narrow alley. The alley measured approximately one meter long, 10 centimeters wide, and 20 centimeters deep. At the end of the alley was a small hole which allowed the mice to escape back into their home cages. Once trained, paper was placed along the alley floor. At the start of a trial, each animal's hindpaws were dipped in nontoxic paint, and the mice were placed at the beginning of the alley and allowed to walk toward their home cages, thus leaving paw prints on the paper underneath (Fernagut et al., 2002; Fleming et al., 2004; Schallert et al., 1978; Tillerson et al., 2002b). Stride length was measured as the distance between ipsilateral hindpaw prints. Only strides made while continuously walking were included in the analyses. Stride lengths at the beginning and the end of the alley were not counted because animals tended to make irregular steps at the beginning as they accelerated, and smaller steps at the end as they slowed to escape the alley (Fleming et al., 2004).

Open field

To assess general locomotor behavior, distance traveled (in centimeters) and average velocity (centimeters per second) were measured in the open field. Behavior was automatically monitored and continuously recorded (Activity Monitor software, version 5) for 20 minutes during each testing session. On each day of testing, mice were placed individually in open field boxes (MedAssociates, St. Albans, VT) and were allowed to move freely. The open field consisted of a sqaure, unobstructed floor measuring 28 centimeters square, surrounded by four, 20 centimeter-high Plexiglas walls. The open field was evenly illuminated inside and was enclosed inside an outer cabinet that measured 64 centimeters wide, 42 centimeters high, and 40 centimeters deep. A small fan built into the cabinet provided low-level white noise to mask extraneous sound. Inside, the floor was criss-crossed

by a grid of infrared beams. When an animal broke two infrared beams, it was scored by the software to have traveled or engaged in a locomotor activity (as opposed to a static motor activity) (Stanford, 2006). Upon completion of the 20-minute session, mice were returned to their home cages. Open field boxes were cleaned of waste then wiped thoroughly with an odor-masking solution (Odormute ^{TM}, Hueter Toledo, Inc., Bellevue, OH, USA) in between testing sessions to ensure removal of olfactory cues left by previous animals.

Striatal DA Analysis

Upon completion of behavioral testing, corresponding to approximately four months after LPS/MPTP treatment, a subset of animals (n=39) were rapidly euthanized and striatal tissue was rapidly dissected (bilateral, 2mm punches from bregma: anterior +0.50, lateral 0.75–2.75; Franklin & Paxinos, 1997) and frozen on dry ice. Striatal tissue samples were placed on dry ice then mailed overnight to Rider University (New Jersey) for high performance liquid chromatography (HPLC) analysis. Samples were coded and processed blind to the treatment conditions. Each sample was removed from –80°C, thawed on ice, and spiked with 50µl of an internal standard of 0.156µM 3,4-dihydroxybenzylamine (DHBA) in cold 0.1M $HClO_4$. Samples were immediately sonicated (5–7 sec, Biologics Model 150V/T Ultrasonic Homogenizer) and centrifuged at 4°C (10 sec, Eppendorf 5415C). Aliquots (20µl) of the supernatants from each sample were manually injected into the HPLC system for catecholamine [DA; dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)] measurements. The HPLC system consisted of a Varian ProStar programmable pump and an Alltech Alltima C18 column (150 mm x 4.6, 5µm) located in a temperature controlled housing (27°C) with an electrochemical detector (Varian Star 9080). The system was operated at a flow rate of 1.0 ml/min with the detector potential set at 0.75V versus an Ag-

AgCl reference electrode. An isocratic mobile phase was used that consisted of 0.33M citrate, 0.67M phosphate (pH 4.5) with sodium octyl sulfate (1.2mM) and 10% methanol. The signal from the electrochemical detector was monitored by a PC computer and processed using Varian Star Chromatography software (version 5). The Varian Star Chromatography software measured area under each peak of DOPAC, DA, and HVA and compared the value obtained to the internal standard. Ten random samples were refrozen and reanalyzed on the HPLC on a second day; the inter-day variability was 8.8% for DOPAC, 2.3% for dopamine, and 7.6% for HVA, respectively. Furthermore, total protein concentration was determined for each sample. Samples were removed from –80°C and thawed to room temperature. The microcentrifuge tubes were spun for 8 minutes and the supernatants removed (and discarded). The remaining pellet was resuspended in 100µl of PBS (pH 7.2) and sonicated (~7 sec). The protein content of each sample was determined using a commercially available protein assay kit (Pierce, Rockford, IL). This assay is based on the bicinchroninic acid method for the colorimetric detection and quantification of total protein. Directions recommended by the supplier for conducting this assay on 96-well plates were followed and the assay reaction was read using a plate reader at 570nm (SpectraCount Microplate Photometer, Packard Bioscience Company, Meriden, CT). Protein content was read off a standard curve of known amounts of bovine serum albumin. Levels (DOPAC, DA, and HVA) were expressed as nanograms per milligram of striatal tissue.

Statistical Analysis

All data were expressed as group means $\pm$ S.E.M. All animals that completed the 16 weeks of behavioral testing were included in the behavioral analyses. Multivariate analysis of variance (MANOVA) was used for all behavioral measures and data were analysed between

groups across 13 total testing sessions. Since there were no differences within treatment conditions, (i.e., in groups with an extra day of injections, as previously described), mice within the same treatments were combined for all analyses, thus groups were saline (n=23), LPS+MPTP (n=51), MPTP-only (n=48) and LPS-only (n=39). The alpha level used for all statistical analyses was 0.05. Tukey's HSD post hoc tests were utilized to further delineate significant univariate ANOVA results for behavioral testing and HPLC data.

Results

All data were expressed as group means $\pm$ S.E.M. All animals who completed the 16 weeks of behavioral testing were included in the behavioral analyses. Multivariate analysis of variance (MANOVA) was used for all behavioral measures and were analysed between groups across 13 total testing sessions. Groups were Saline (n=23), LPS+MPTP (n=51), MPTP only (n=48) and LPS only (n=39). Since there were no differences within treatment conditions between doses, (in groups with an extra day of injections, as previously described in the Method section), mice within the same treatments were combined for all analyses. The alpha level used for all statistical analyses was .05. Tukey's HSD post hoc tests were utilized to further delineate significant univariate ANOVA results for behavioral testing and HPLC data.

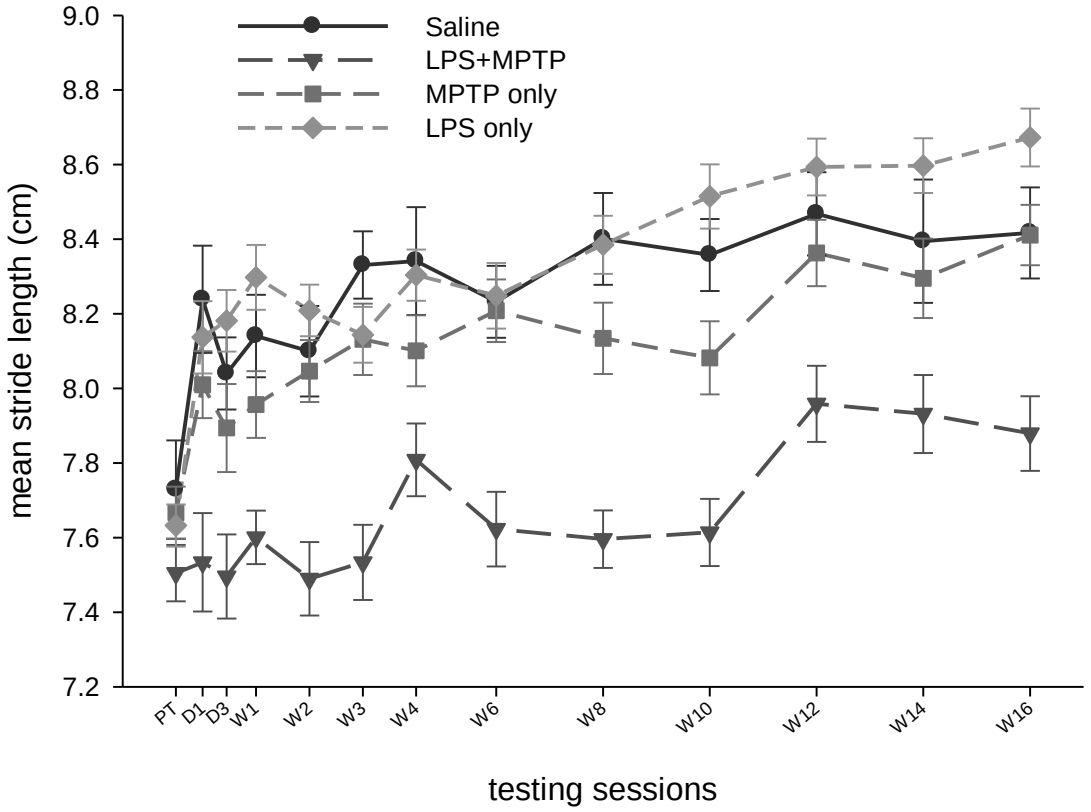

Figure 1. Mean stride length in centimeters of C57BL/6J mice receiving either saline, LPS+MPTP, MPTP-only, or LPS-only. There was an overall significant treatment effect on mean stride length; mice in the LPS+MPTP group showed significantly reduced mean stride lengths when compared to all other groups with the exception of the first, pretest session. Please refer to Table 2 for means, univariate statistics and post hoc results for each testing session. Note: D – Day; W – Week.

Stride length

As shown in Figure 1, mice in the LPS+MPTP group showed overall reductions in mean stride length compared to saline, MPTP-only, and LPS-only mice in all behavioral testing sessions with the exception of the pretest session. Mean stride length was measured as the distance between hindlimb pawprints on the same side of the body (in centimeters) and

was used as the dependent variable in a 4 (LPS-MPTP treatment condition) X 13 (testing sessions) MANOVA which showed a significant effect of treatment condition on mean stride length, $F(39,441) = 2.653$, $p < 0.05$. Further univariate analysis tests between groups within each testing session showed significant differences in mean stride length with the noted exception of the pretest; univariate statistics are detailed in Table 2 along with group mean stride lengths and post hoc results.

Table 2. Mean stride length (in centimeters) and univariate statistics for each of 13 stride length testing sessions in C57BL/6J mice receiving either saline, LPS+MPTP, MPTP-only, or LPS-only.

	Saline	LPS+ MPTP	MPTP only	LPS only	*F*	sig
Pretest	7.73(.103)^{a}	7.51(.070)^{a}	7.67(.072)^{a}	7.63(.080)^{a}	1.417	*ns*
D1	8.24(.155)^{b}	7.53(.104)^{c}	8.01(.107)^{b}	8.14(.119)^{b}	7.328	.000
D3	8.04(.149)^{d}	7.50(.099)^{e}	7.89(.102)^{d}	8.18(.113)^{d}	7.722	.000
W1	8.14(.114)^{fg}	7.60(.078)^{h}	7.96(.080)^{f}	8.30(.089)^{g}	12.696	.000
W2	8.10(.124)^{i}	7.49(.083)^{j}	8.05(.085)^{i}	8.21(.095)^{i}	13.572	.000
W3	8.33(.124)^{k}	7.53(.086)^{l}	8.13(.088)^{k}	8.14(.098)^{k}	13.441	.000
W4	8.34(.131)^{m}	7.81(.088)^{n}	8.10(.091)^{mn}	8.30(.101)^{m}	6.130	.001
W6	8.23(.127)^{o}	7.62(.085)^{p}	8.21(.087)^{o}	8.25(.097)^{o}	11.599	.000
W8	8.40(.121)^{q}	7.60(.081)^{r}	8.13(.083)^{q}	8.39(.093)^{q}	18.018	.000
W10	8.36(.126)st	7.61(.085)^{u}	8.08(.088)^{s}	8.52(.097)^{t}	18.196	.000
W12	8.47(.128)^{v}	7.96(.086)^{w}	8.36(.089)^{v}	8.59(.098)^{v}	3.378	.000
W14	8.38(.130)^{x}	7.93(.089)^{y}	8.30(.091)^{x}	8.57(.101)^{x}	9.041	.000
W16	8.42(.105)^{z}	7.82(.075)^{aa}	8.41(.077)^{z}	8.66(.085)^{z}	21.453	.000
change†	.799(.132)^{bb}	.395(.127)^{cc}	.919(.148)^{bb}	1.10(.104)^{bb}	10.147	.000

Note. D – Day; W – Week; values represent mean stride length (±S.E.M.). Superscripts denote Tukey's post hoc significant differences between groups within testing sessions ($p<.05$). Overall MANOVA showed mean stride lengths differed significantly ($p<.05$) between groups. The error *df* for all univariate analyses was 157; † denotes the change, in centimeters, from pretest to the final testing sessions on Week 16 for each group (i.e., a positive number indicates an increase in stride length); post hoc data show the increased stride length in mice in the LPS+MPTP group from pretest to Week 16 was significantly less than any other groups, none of which differed from each other.

Generally, across all but pretest testing sessions, mice in the LPS+MPTP group showed significantly reduced mean stride length when compared to saline, MPTP-only, and LPS-only mice. Additional reductions in mean stride length were shown by the MPTP-only group, which had shorter mean stride lengths when compared to LPS-only mice during Weeks 1 and 10.

A second univariate ANOVA was conducted on the change (in centimeters) in stride length from pretest to the final testing session (Week 16), in order to ascertain whether the increases seen in stride length over time, across the 13 testing sessions, were the same for all groups. There was a significant effect of treatment condition on the mean increase or change in stride length across testing sessions, $F(3,157) = 10.174$, $p < 0.05$. Tukey's post hoc analyses showed the mean increase in stride length in mice in the LPS+MPTP group across testing sessions was significantly less than mice in either the saline, MPTP-only, or LPS-only groups, which did not differ from one another. Please refer to Table 2 for mean change in stride length means.

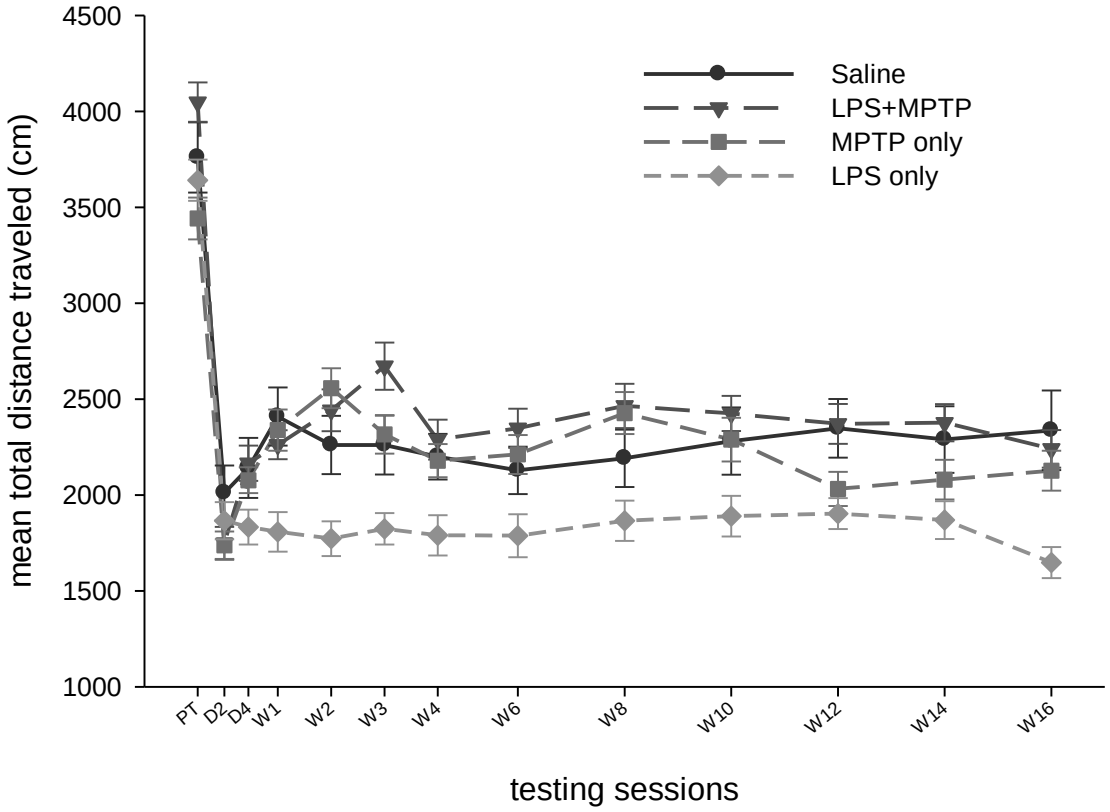

Figure 2. Mean distance traveled in centimeters during a 20-minute open field session by C57BL/6J mice receiving either saline, LPS+MPTP, MPTP-only, or LPS-only. There was an overall significant effect of treatment condition on mean total distance traveled in the open field; mice in the LPS-only group generally traveled significantly less distance during a 20-minute open field session when compared to mice in saline, LPS+MPTP, and MPTP-only groups. Further analyses on mean velocity showed no group differences; therefore the reduction in distance traveled was not due to decreased speed. Please refer to Table 3 for distance traveled means, univariate statistics, and post hoc results for each testing session. Note: D – Day; W – Week.

Open field: Distance traveled

As shown in Figure 2, mice in the LPS-only group showed overall reductions in mean total distance traveled in the open field when compared to saline, LPS+MPTP, and MPTP-

only groups in all behavioral testing sessions. Mean distance traveled in centimeters was calculated for all groups as recorded during a 20-minute open field session and was used as the dependent variable in a 4 (LPS-MPTP treatment condition) X 13 (testing sessions) MANOVA. There was an overall significant effect of treatment condition on mean total distance traveled in the open field, $F(39,441) = 2.653$, $p < 0.05$. Univariate analysis tests between groups within testing sessions showed significant differences in mean total distance traveled in all testing sessions with the exception of post-injection Days 2 and 4; univariate statistics are detailed in Table 3 along with group mean total distance traveled and Tukey's post hoc results.

Table 3. Means for total distance traveled (in centimeters) in the open field and univariate statistics for each of 13, twenty-minute open field sessions of C57BL/6J mice receiving either saline, LPS+MPTP, MPTP-only, or LPS-only.

	Saline	LPS+ MPTP	MPTP only	LPS only	*F*	sig
Pretest	3759(182)^{ab}	4047(102)^{a}	3441(108)^{b}	3640(107)^{ab}	5.681	.001
D2	2010(143)^{c}	1748(85)^{c}	1737(71)^{c}	1865(97)^{c}	1.424	*ns*
D4	2140(156)^{d}	2165(92)^{d}	2076(67)^{d}	1832(90)^{d}	2.539	*ns*
W1	2408(151)^{e}	2261(75)^{e}	2338(107)^{e}	1807(103)^{f}	6.259	.000
W2	2260(152)^{g}	2441(108)^{g}	2556(103)^{g}	1772(90)^{h}	10.047	.000
W3	2260(154)^{ij}	2671(123)^{i}	2315(100)^{i}	1823(81)^{j}	10.030	.000
W4	2199(119)^{k}	2288(103)^{k}	2178(86)^{kl}	1789(104)^{l}	4.641	.004
W6	2128(123)^{mn}	2347(102)^{m}	2211(101)^{mn}	1787(112)^{n}	4.991	.004
W8	2191(149)^{op}	2464(115)^{o}	2426(109)^{o}	1865(105)^{p}	5.659	.001
W10	2280(175)^{qr}	2424(91)^{q}	2288(113)^{qr}	1889(106)^{r}	4.221	.007
W12	2347(153)^{s}	2370(103)^{s}	2031(88)st	1902(80)^{t}	5.041	.002
W14	2289(173)^{uv}	2376(97)^{u}	2079(103)^{uv}	1869(98)^{v}	4.275	.006
W16	2336(207)^{w}	2240(97)^{w}	2126(104)^{w}	1647(80)^{x}	6.654	.000

Note: Values represent mean distance traveled (±S.E.M.) in centimeters. Superscripts denote Tukey's post hoc significant differences between groups within testing sessions ($p<.05$). Overall MANOVA showed mean distance traveled differed significantly ($p<.05$) between groups. The error *df* for all univariate analyses was 157.

Generally, across all testing sessions, mice in the LPS-only group traveled significantly less distance during a 20-minute open field session when compared to mice in saline, LPS+MPTP, and MPTP-only groups.

Open field: Average Velocity

To ascertain whether the differences found in mean distance traveled in the open field were due to slower movement, mean average velocity in centimeters per second was calculated for all groups and was used as the dependent variable in a 4 (LPS-MPTP treatment condition) X 13 (Testing Sessions) MANOVA. There was not a significant effect of treatment condition on mean average velocity in the open field, $F(39,441) = 1.318$, *ns* (data not shown).

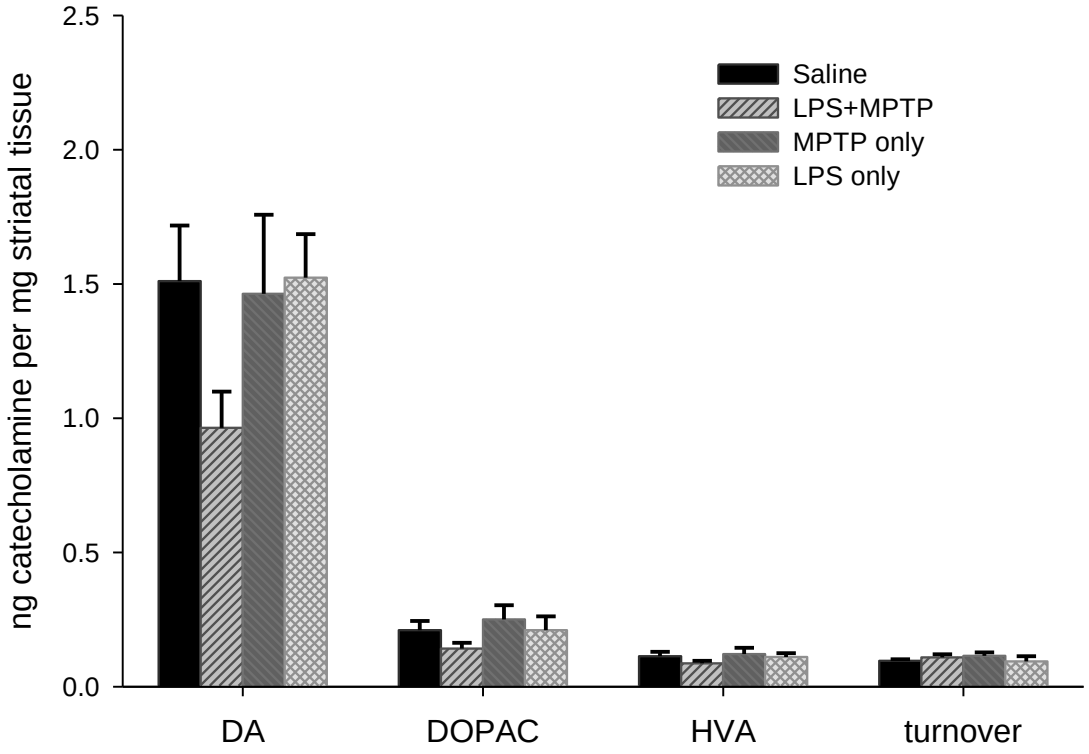

Figure 3. Effects of LPS (3x 250µg/kg) plus MPTP (15mg/kg) on striatal levels of DA at four months postinjection in mice receiving saline (n= 8), LPS+MPTP (n=13), MPTP-only (n=7), or LPS-only (n=11). Post hocs showed mice in the LPS+MPTP group had significantly lower levels of striatal DA when compared to mice in either the saline or LPS-only groups. There was so significant effect of treatment condition on levels of either DOPAC or HVA nor on the turnover ratio.

Striatal DA

HPLC analysis was used to determine levels of intracellular striatal DA and its metabolites (DOPAC, HVA) as well as the metabolite turnover ratio [(DOPAC+HVA)/DA; Petzinger et al., 2007] in a subset of mice upon completion of behavioral testing (approximately four months post-injection). For all HPLC dependent measures, univariate ANOVAs were conducted between the four groups (LPS-MPTP treatment condition) with

levels of striatal DA, DOPAC, and HVA, as well as the turnover ratio as dependent variables. As seen in Figure 3, there was a significant effect of treatment condition on levels of striatal DA per milligram of tissue, $F(3,35) = 4.974$, $p < 0.05$. Tukey's post hoc analyses further showed mice in the LPS+MPTP group had significantly less DA in bilateral striatal tissue punches than mice in the saline and LPS-only groups, $p < 0.05$. Also, there was no significant effect of treatment condition on either DOPAC, $F(3,35) = 2.460$, *ns,* or HVA, $F(3,35) = 2.470$, *ns*, nor on the turnover ratio, $F(3,35) = .710$, *ns*.

Discussion

The present study represented a first attempt to evaluate the long-term, behavioral and neurochemical effects of systemic, bacterial endotoxin (LPS) plus systemic MPTP as an *in vivo* model of PD in the C57BL/6J mouse. In agreement with previous research utilizing either 6-OHDA or MPTP rodent models of PD (Amende et al., 2005; Fernagut et al., 2002; Schallert et al., 1978; Tsai et al., 1991), animals receiving LPS+MPTP were found to have significantly reduced stride length compared to mice in either the MPTP-only, LPS-only, or saline groups. This finding is also consistent with reductions in stride length that are characteristic of clinical PD (Hausdorff et al., 1998; Nieuwboer et al., 2001). Moreover, in contrast to previous reports that parkinsonian behavioral deficits were absent or transient (Willis & Donnan, 1987; Jakowec et al., 2004), the reduction in stride length exhibited by LPS+MPTP mice in the present study was consistent and long-lasting, persisting for at least four months post–injection. Concurrent with stride length deficits, striatal DA levels remained significantly reduced at the end of the four-month behavioral testing period in LPS+MPTP mice (Figure 3). These data are consistent with previous work that found reductions with combinations of LPS and MPTP either *in vitro* (Gao et al., 2003a) or by

intracerebral infusion (Goralski & Renton, 2004). With the addition of LPS to the MPTP mouse model, the long-term reduction in striatal DA in the LPS+MPTP group stands in contrast to other reports that striatal DA recovers by as soon as day 28 post-administration with MPTP alone, even despite doses higher than that used in the present study (e.g., Arai et al., 1990; Ogawa et al., 1987; Petzinger et al., 2007). Furthermore, contrary to previous work with relatively higher doses of MPTP in C57BL/6J mice (Sundström et al., 1990; West et al., 2006), mice in the present LPS+MPTP group showed no deficits in locomotor activity in open field measures, neither in distance traveled nor average velocity. Since the co-administration of LPS and MPTP produced no mortality and the LPS+MPTP group were not bradykinetic in the open field, it may be further concluded that the combination of LPS and MPTP was not toxic enough to produce hypokinesia or general sickness behavior. Rather, it may be that the reduction in stride length exhibited by this group was reminiscent of the shortened stride length seen in PD patients (e.g., Nieuwboer et al., 2001).

The effects of LPS and/orMPTP treatment on striatal levels of DA and its metabolites were assessed at the conclusion of behavioral testing (four months; Figure 3). At the four-month time point, mice receiving MPTP-only showed no reductions in striatal DA. These data are in line with those that reported MPTP produced transient DA damage that recovered (Arai et al., 1990; Ricaurte et al., 1986; Tillerson et al., 2002b).

Behaviorally, a short, high-dose MPTP regimen can lead to marked, long-term deficits in locomotor activity (Sundström et al., 1990). Other research with mice given MPTP in the open field showed reduced total mean distance traveled and distance decreased as the dose (40, 60, and 80 mg) of MPTP increased (West et al., 2006). However, behavioral deficits were not expected with a low, 15 milligram dose of MPTP. These results are

concurrent with others that reported no behavioral deficits with MPTP (Gupta et al., 1986; Willis & Donnan, 1987). Therefore, in the MPTP mouse model, it may be that the detection of behavioral abnormalities is highly dependent upon the MPTP dosing regimens and sensitivity of the behavioral measures, and thus the variation across studies in experimental design may be responsible for the spectrum of often contradictory results.

LPS-only mice showed a persistent and consistent depression in distance traveled in the open field compared to saline, LPS+MPTP, MPTP-only mice for the duration of the post-injection period. However this group did not show any reductions in overall average velocity, which indicates that the reductions seen in distance traveled were not due to decreased speed of travel. Therefore, it seems that the reduction in distance traveled is not necessarily indicative of a motor deficit, *per se*. Also, these data represent a novel and unexpected finding. For instance, Sparkman et al. (2005) showed, when motivated by mild footshock (0.4mA) in a two-way active avoidance paradigm, locomotor behavior in mice receiving doses of LPS comparable to the one used in the present study recovered to that of controls in a very short period of time (i.e., a few days). In fact, locomotor behavior actually increased over controls as measured by the number of intertrial-interval (ITI) crossings (ITI crossings are an indicator of general locomotor behavior) (Sparkman et al., 2005). LPS-only mice did not exhibit any gait impairment when examined in the stride length test. Also, contrary to reports of increased DA cell loss in adulthood after prenatal exposure (Carvey et al., 2003); *in vitro* reports of DA degeneration in mesencephalic neuron-glia cultures (Gao et al., 2002b), and selective destruction of SNc neurons after intranigral infusion (Castano et al., 1998; Iravani et al., 2005), LPS alone did not induce reductions in striatal DA in the present study. Therefore, these preliminary data do not suggest that inflammation alone is capable of

inducing long-term deficits that are typically or uniquely parkinsonian in nature (Hirsch et al., 2003; Iravani et al., 2005; Perry, 2004), yet this apparent contradiction with previous LPS work using prenatal exposure or intranigral infusion (e.g., Carvey et al., 2003; Castaño et al., 1998) perhaps warrants further exploration.

Overall, the present study offers support to the assertion by Gao et al. (2003a) that LPS followed by MPTP produces a kind of 'synergistic' toxicity. It also supports previous research which suggested that factors produced in the inflammatory cascade enhance the toxic effects of MPTP and serve to increase dopaminergic cell damage (Wu et al., 2003; Teismann et al., 2003; Vijitruth et al., 2006). Furthermore, it seems by using LPS in combination with MPTP, it may be possible to reproduce parkinsonian behavioral deficits in mice by using much lower doses of MPTP than are typically employed. For instance, in Amende et al. (2005), MPTP mice had a significantly reduced mean stride length of about 6.6 centimeters $\pm$ 0.1 after a total MPTP dose of 90 milligrams. Mice in the present study in the MPTP-only condition averaged longer stride lengths of 8.15 centimeters, while mice receiving both LPS+MPTP group averaged significantly shorter lengths of about 7.6 centimeters for the duration of the four month post-injection period (Figure 1). Moreover, the present data are in agreement with previous research which also found chronically decreased stride lengths in research utilizing 6-OHDA toxin-induced rodent models of PD (Schallert et al., 1978). The present model also addresses several concerns raised by those who have investigated the MPTP mouse model (Beal, 2001; Ogawa et al., 1987; Orth & Tabrizi, 2003; Schober, 2004; Schmidt & Ferger, 2001). For instance, it has been believed that the MPTP primate model was the only suitable way in which to utilize MPTP to examine parkinsonian behavioral deficits. The reason for this was because, as previously described, any behavioral

deficits reported in mice tended to be only transient and reversible short-term, toxicity effects (Przedborski et al., 2001; Schober, 2004).

The differences in distance traveled in the open field between LPS+MPTP and LPS-only mice are somewhat perplexing. Perhaps it is important to recognize that the two behavioral tests in the present study differed markedly in their respective locomotor requirements. In the open field, movement is normally casual, meandering, and undirected. Conversely, in the stride length test, movement is comparatively explosive, dynamic, and directed. Most importantly, in the stride length test, the mice have an escape (unlike the open field) (Rodgers, 2007). Clinically, in PD patients, the demands of the movement task influence the movement itself. It is known that as movement requirements become faster, more intense, and more accelerating, patients' gait disturbances increase and their steps become shorter, sometimes to the point of falling (festinating gait) (Nieuwboer et al., 2001). Therefore, it seems reasonable to surmise that factors (such as escape potential or acceleration requirement) affecting movement in one test might not necessarily be important in the other.

The present study also offers an opportunity to investigate the potential role of stress in PD pathogenesis. Although the relationship between stress and the immune system is complex and incompletely understood, stress may play a causal role in PD and other neurological disorders (e.g., schizophrenia, Alzheimer's) (Ishihara & Brayne, 2006; Kronfol & Remick, 2000; Smith et al., 2002; Walsh & Bennett, 2001). Stress may then render an organism more vulnerable to exogenous toxin exposure. Braak et al. (2003b) have shown how an exogenous agent could be transported into central structures gaining entrance via the enteric nervous system and if the organism is inflamed and/or stressed, the toxic effects could

be increased. Experimental research in rats has shown that when first exposed to environmental stress, the toxic effects of 6-OHDA administration on the dopaminergic system were significantly increased compared with rats who never experienced the environmental stressors (Keefe et al., 1990). Others found the protective effects of exercise on the DA system were negated by environmental stress in the 6-OHDA rat model (Faherty et al., 2005; Fisher et al., 2004; Howells et al., 2005; Kleim et al., 2003; Mabandla et al., 2004; O'Dell et al., 2007; Poulton & Muir, 2005; Tillerson et al., 2003).

Conclusion

It has become apparent from a number of lines of research, not just in PD, that infections of peripheral origin can have a profound impact on the survival of central neurons (Combrinck et al., 2002; Cunningham et al., 2005; Perry, 2004). The addition of LPS to the MPTP C57BL/6J mouse model of PD in an *in vivo*, systemically-administered paradigm supports the idea that parkinsonian neurodegeneration may begin early, perhaps outside of the nigrostriatal dopaminergic system (Braak et al., 2003a,b; Del Tredici et al., 2002; Lang, 2007; Lang & Obeso, 2004).

It remains important to evaluate this model at various time points neurochemically to ascertain precisely how the LPS+MPTP combination affects striatal DA or its metabolites at various time points after administration. In addition to information on catecholamine content, it would be advantageous to evaluate the present model for central inflammatory activity, i.e., microglia proliferation (Kohutnicka et al., 1998), central proinflammatory cytokine expression (Szelényi & Vizi, 2007), and alpha synuclein aggregation (Fleming et al., 2004). It would also be advantageous to evaluate this model in aged mice. Previous research with MPTP or LPS alone has shown that aged mice receiving LPS are more adversely affected

than young animals (Kohman et al., 2007a) and after MPTP, aged mice have neuropathology that is more widespread than young mice, which is more representative of the pathology seen in aged, idiopathic PD patients (Gupta et al., 1986). Recently the synergistic effects of neonatal iron overload and MPTP on the integrity of dopamine neurons was found to be detectable only during old age (Peng et al., 2007). Moreover, early compromise of dopamine and related neurons associated with mild, transient deficits during young adulthood can lead to the re-emergence of deficits during old age (Schallert, 1983, 1988). Finally, animal models of PD that mimic end-stage DA depletion and pathology remain important tools for the investigation of treatment for advanced PD patients (e.g., Schallert et al., 1979). However, in addition to providing preliminary behavioral and neurochemical data consistent with PD, the present LPS+MPTP C57BL/6J mouse model holds advantages for the evaluation of early PD that include use of (theoretically) causally-oriented toxic agents (e.g., Braak et al., 2003b; Lang, 2007; Whitton, 2007), relative cost effectiveness (Jakowec & Petzinger, 2004), and simple, systemic administration methods.

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PROFESSIONAL MEMBERSHIPS

- Society For Neuroscience
- International Behavioral Neuroscience Society
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ABSTRACT

SYSTEMIC BACTERIAL ENDOTOXIN PLUS MPTP AS A MODEL OF PARKINSON'S DISEASE IN C57BL/J6 MICE

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The integration of recent, multifactor theories of Parkinson's disease (PD) etiology into animal models of the disease comprise a relatively small portion of the research. In most environmental models of PD, a single neurodegenerative agent is introduced to cause nigrostriatal dopamine depletion. It has been argued, however, that cell loss in human PD often might derive, at least in part, from multiple toxins or vulnerabilities, any one of which alone does not lead to chronic dopamine depletion. Based on previous *in vitro* research, two agents were delivered to mice peripherally to promote chronic dopamine depletion and neurological impairment: the inflammatory bacterial endotoxin, lipopolysaccharide (LPS) and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Male C57BL/6J mice received treatment with LPS+MPTP, MPTP-only, LPS-only, or saline and then were evaluated for four months on stride length and motor function in an open field. Mice in the two-factor (LPS+MPTP) group, but not in the single-factor groups, showed dopamine depletion and impaired motor function, including reduced stride length at four months post-

injection. The LPS- and MPTP-only groups showed no dopamine depletion or parkinsonian, behavioral deficits. These data are consistent with the view that nigrostriatal dopamine neurons conceivably might succumb chronically to multiple toxic agents that independently may have only a transient adverse effect. In addition to providing preliminary behavioral and neurochemical data consistent with PD, the present LPS+MPTP C57BL/6J mouse model holds advantages for evaluation of early PD that include use of (theoretically) causally-oriented toxic agents, relative cost effectiveness, and simple, systemic administration methods.