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Review

The gestational environment and Parkinson's disease: Evidence for neurodevelopmental origins of a neurodegenerative disorder

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Abstract

Parkinson's Disease (PD) is a degenerative neurological disorder that typically manifests symptoms in late adulthood, after loss of dopaminergic neurons in the nigrostriatal system. A lack of heritability for idiopathic PD has implicated adulthood environmental factors in the etiology of the disease. However, compelling evidence from animal models published within the past few years has shown that a range of environmental factors occurring during the perinatal period (including exposure to the common pesticides paraquat and maneb, organochlorine pesticides, and iron-enriched diet) and the prenatal period (including the pesticide maneb, cocaine, and the bacterial product LPS) can either directly cause a reduction in the number of dopamine neurons, or cause an increased susceptibility to degeneration of these neurons with subsequent environmental insults or with aging alone. In this review, these models are described for potential relevance in linking PD with the Fetal Basis of Adult Disease (FeBAD) hypothesis. Additionally, challenges in studying the neurodevelopmental basis of neurodegeneration experimentally and epidemiologically are presented.

Keywords: (*n* = 8) Fetal basis of adult disease; Parkinson's disease; Maneb; Paraquat; Multiple hit hypothesis; Silent toxicity; Nigrostriatal system; Dopamine

Contents

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder clinically characterized by bradykinesia, rigidity, resting tremor and postural instability. PD is pathologically marked by depletion of dopaminergic (DAergic) cell bodies in the substantia nigra *pars compacta* (SNpc) with subsequent loss of DA in the nigrostriatal system, and the presence of Lewy bodies. Estimates of prevalence of PD have ranged between less than 100 to more than 300 per 100,000 people (for review, see ref. [1]), and PD has recently become one of the top 15 leading causes of death in the US [2]. PD is most commonly associated with the aging population, and the onset and peak incidence of the disease occurs in the sixth decade of life [3]. Although genetic factors have been found to play a role in some instances of the disease, environmental factors such as pesticide exposures have been widely implicated in the etiology of idiopathic PD, via clinical case reports, epidemiological surveys, and animal models (for example refs. [4–11]). Because PD is associated with a late-in-life onset, research has traditionally considered PD to be a disease of aging, despite a long history of speculation that environmental factors early in life can predispose or even cause an individual to develop Parkinsonian symptoms [12–17]. Until the past few years, experimental evidence has been lacking to support this hypothesis. Recent reports, however, have begun to identify specific factors occurring as part of the in utero or perinatal environment that may cause or predispose the nigrostriatal system to damage, and these factors may warrant us to reconsider at least some instances of idiopathic PD to be the consequence of a neurodevelopmental disruption, rather than strictly a consequence of aging.

1.1. Developmental origins of adult neurologic dysfunction

An emerging scientific shift toward studying the developmental basis of health and disease (or, the fetal basis of adult disease (FeBAD)) is based on the finding that susceptibility to certain diseases can be established by environmental stressors encountered very early in life (in utero or perinatally) [18]; the majority of studies testing this model, to date, have focused on skewed nutritional states, with examination of outcomes including cardiovascular disease, diabetes and cancers [19,20]. Though few models have considered neurodegenerative disorders like PD, the idea that the fetal environment can hold implications for adulthood neurologic disease is based on solid rationale and some model systems [21]. The most thorough example studying this relationship perhaps comes from the study of schizophrenia, where interactions between genetics and early-life environment, including prenatal lead exposure, first trimester influenza infection and nutritional factors (for example, the Dutch Famine) have been implicated in disease etiology [22]. This example of FeBAD posits that, in the right genetic background, one or more of these pathogenic events occurring during brain development will result in a static brain lesion, though the behavioral consequences of this event remain latent until a later-in-life causative "stress" leads to the emergence of symptoms [23]. In another potential example of neurological FeBAD, perinatal exposure to lead in rats caused lifelong consequences on expression of proteins associated with Alzheimer's disease (AD) [24]; other early-life factors that may play a role in AD have been determined epidemiologically and include large sibship, area of residence and socioeconomic status, though the role of these factors during gestation has not been studied [25,26].

These examples demonstrate that a fetal basis to later-onset neurological disease is not an unprecedented hypothesis; there are, however, some unique challenges in studying gestational environmental factors as they relate to the FeBAD hypothesis and PD. One of the most significant challenges is the issue of silent toxicity and the tremendous ability of the developing nervous system to compensate for insults. These challenges were articulated by Reuhl [27], describing silent toxicity as being a persistent morphological and/or biochemical injury that remains clinically unapparent unless unmasked. Through compensatory mechanisms, the affected system initially appears normal when considered against traditional biomarkers for toxicity (such as gross morphological and histological examination, behavioral assays or mortality studies), though the system is functionally compromised [18]. It is hypothesized, therefore, that silent toxicity induces a state of "altered potential," or a "mutant steady state," where abnormal homeostasis (associated with increased vulnerability and higher risk of cell death) is established in a time-, tissue- and toxicant-specific manner, is maintained by altered gene expression, and is persistent and irreversible [18,19,28]. Though it presents a major challenge in identifying causative exposures, it is important to emphasize that silent toxicity is non-teratogenic, and should not directly cause observable behavioral or psychological effects until after an unmasking event. Unmasking challenges can be physical or toxicological exposures, pathophysiological strains (such as disease, stress or malnutrition) or even endogenous events like normal

development and aging; these factors can be long-lasting and progressively damaging [21,27,29]. There may be an extremely long latent period following the adverse environmental condition [30] (including trans-generational latency [31]) in which silent toxicity is maintained before the unmasking event(s); the end effect of an unmasking insult is always the breach of boundaries of compensation, with the subsequent push on the neurological system beyond some clinical threshold [21,27]. Ultimately, the consequence of the gestational exposure coupled with the unmasking challenge can be the onset of disease that otherwise would not have occurred, an increase in risk of disease, an earlier onset of disease and/or an increased severity of the disease [19]; these scenarios present a working model in which to study the FeBAD hypothesis as it relates to PD.

1.2. Parkinson's disease and the multiple hit hypothesis

There are features of this basic outline that are especially relevant to studying PD as a consequence of multiple environmental insults (including gestational exposures). First, the concept of PD as a "threshold" disease has been widely accepted for decades, originally based on extrapolation from human postmortem samples (for example refs. [32,33]) and more recently supported by regression analysis in a primate model of PD [34]. The threshold concept states that clinical symptoms of PD will only appear after DAergic function has been diminished by a set percentage of "maximal function," and that this threshold usually does not occur until late-in-life; traditionally, according to the Calne and Langston model (1983), this is an 80% loss [35], though there is compelling evidence suggesting a much less profound loss and inter-individual variances in threshold [36]. Implicit within this model of PD is that, with the exception of some immediate acute event that pushes the nigrostriatal system to this threshold (for example, individuals who unknowingly injected themselves with the DAergic toxicant MPTP [37]) there is a long period (lasting years or decades) in which the nigrostriatal system is operating at a submaximal level, but in which there are no Parkinsonian symptoms to suggest the presence of disease until that threshold is met [38–41].

A further assertion of this basic model is that decline in DAergic function (which would bring the system closer to threshold) is a slow and unremitting part of natural aging (see original hypothesis [35], which has been extensively described, reproduced and/or modified [13,17,27,36,39,42–48]). However, there are equivocal reports on the role of normal aging in the nigrostriatal system, and significant human studies and animal models have de-emphasized a role of aging in isolation from other factors, via absent evidence of cell loss and/or diminished DAergic function with aging (for example refs. [49–56]). Old age, as it relates to PD, may simply be the point in time where other risk factors culminate and interact sufficiently to cause disease [57], thus yielding the typical adulthood onset of PD near the sixth decade of life. The absence or diminution of a significant role for "normal aging" (as a natural process in the continuum of development across the lifespan) in the etiology of PD thus shifts attention onto early, persistent, cumulative and/or progressive environmental factors in the etiology of the disease, and this presents a second important feature of this disease that makes it amenable to the "multiple hit hypothesis." This hypothesis accounts for issues like silent toxicity, latency, unmasking and threshold by stating that a neural system may be able to compensate for damage induced by one environmental exposure ("hit"), but when multiple hits occur to the same target (especially via different mechanisms and/or at different developmental stages), re-regulation is not possible, vulnerability increases, homeostasis fails, threshold is breached and clinical symptoms emerge (Fig. 1) [44].

The rate at which DAergic dysfunction in PD occurs is still unclear. Various models have suggested either that individual hits accelerate the decline of DAergic function (Fig. 1A), that acute adverse effects followed by a return to a normal rate (thus operating at a lower but parallel curve; Fig. 1B), or that some mixture of these two models occurs (Fig. 1C). To date, most experimental studies related to PD have focused on causing or preventing DAergic dysfunction during or immediately following an acute toxic insult [13]. Indeed, it has been proposed that to maintain chronic and progressive neurodegeneration, an environmental toxicant must be continually present [42]. However, some recent studies have detected progression across long time periods after acute or chronic toxicant exposure (for example refs. [54,58–61]), thus supporting the hypothesis that "an environmental insult that is not continuous could provoke a progressive disease that is" [58]. While a complete set of evidence is still lacking, this basic multiple hit model provides additional features to use in studying the role of critical developmental periods for silent toxicity, combined with environmental insults occurring from gestation through old age, as risk factors for the development and emergence of Parkinsonian symptoms.

2. Developmental risks in Parkinson's disease

2.1. Infectious etiology in human populations?

Historically, the developmental risk factor for PD that initially garnered the most attention was viral infection, based on the disease clusters that occurred following clinical encephalitis or influenza (von Economo disease, or encephalitis lethargica (for review, see refs. [62,63])). An epidemiological study sought to establish a delayed development of PD later in life (rather than the more immediate syndrome characteristic of von Economo's), by attempting to link young-adulthood influenza infection during epidemic years to late-onset PD [16]. Conducted over 40 years ago, it was a limited study finding weak associations, though it did importantly outline many of the basic features that more recent models have tried to establish, including an early-life insult, a decades-long latent period (during which, it was originally hypothesized, an infectious agent could progressively destroy cells) and the eventual emergence of disease. Twenty-five years following that report, a study in the UK examined a role for an even earlier point in development (gestation). Based on the hypothesis that influenza was toxic to the developing substantia nigra, it was suspected that individuals incurring this risk factor in utero would be born with a limited reserve of DAergic neurons and therefore be more vulnerable to developing PD

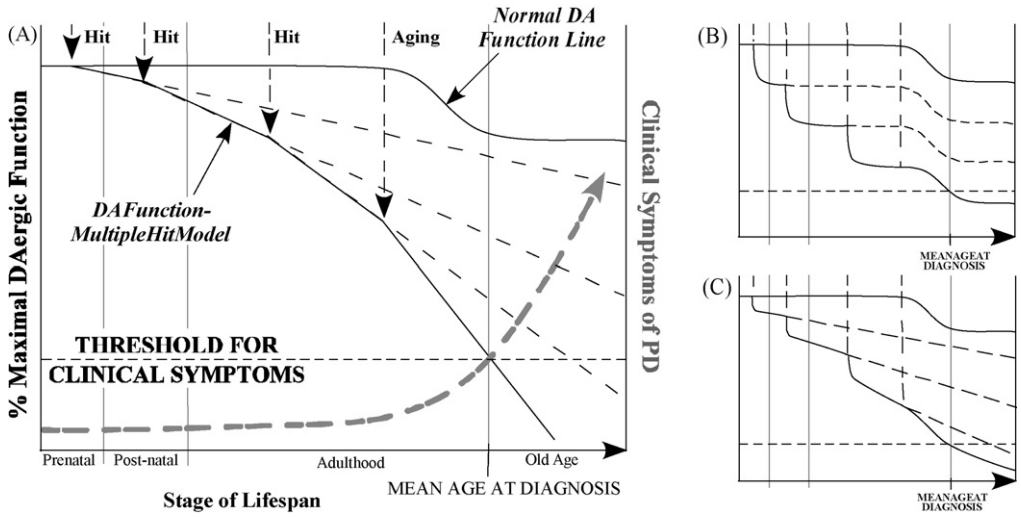

Fig. 1. The multiple hit model of PD (based on ref. [35]). (A) Different developmental periods occur across the lifespan (*x*-axis), with the typical onset of idiopathic PD occurring about the sixth decade. The left *y*-axis represents a theoretical measure of integrity of the DAergic system (percent of maximal function) and there is a threshold level of this DAergic function, which, when breached, results in clinical symptoms. The upper solid curve (normal DA function line) models a state in which DA function remains near maximal for the duration of the lifespan, with some degree of decline as the individual ages (while the physiological role of aging on the DA system is equivocal [49–56], we here represent normal aging to play some role in DAergic decline). The lower solid curve represents DA function in the multiple hit model of PD, where "hits" occurring across the lifespan combined with aging (vertical dashed lines + arrowheads) are each capable of inducing a steeper slope of decline that implies progression of vulnerability (solid lines extended with dashed lines); the cumulative effect of subsequent hits occurring at different stages across the lifespan is an overall acceleration of the curve toward threshold. As discussed in the text, hypothesized hits may include LPS, cocaine, maneb, paraquat, rotenone, dieldrin, infections or altered nutritional states [12,16,17,31,61,69,86–88,96]; though for simplicity the effects of hits are drawn only on a sequential and cumulative course, each individual hit is capable of causing a downward deviation, independent of preceding events and the deviations may interact with and exacerbate preceding events; see (C). The right-side *y*-axis represents clinical symptoms of PD, and is described by the dashed gray curve. As DA function in the multiple hit model accelerates toward threshold, there is a prolonged period during which DA function is grossly sub-maximal, but symptoms of PD are unapparent [38]. When threshold is breached, there is a sharp increase in the severity of Parkinsonian symptoms, which continue to increase as the disease progresses; this breach is indicated at the "mean age at diagnosis," where the lines of threshold, DA function in the multiple hit model and clinical symptoms all intersect. (B) In a variation of (A) (some graphic features removed for simplicity), each individual hit is associated with immediate, acute and discrete adverse effects on DAergic function, after which a curve parallel to but lower than normal is assumed (dashed lines); this model is more punctuated, and implies an absence of progression of vulnerability and lack of interaction with preceding hits. (C) In a combination of the models in (A and B), hits are both punctuated and progressive; a hit may induce an immediate adverse drop in the system, followed by an alteration in the subsequent slope of decline. Subsequent hits interact with preceding insults and the magnitude of decline (slope) increases with each hit. In all three models, additional potential modifying factors were not graphically presented, but have significant implications on the response to hits, including gender [45] and genetic background [134,135].

later in life [64]. Histograms of PD incidence arranged by birth-year indeed did often correlate to years of influenza pandemic (e.g. 1892, 1918–1919 and 1929), suggesting clustering of cases around a significant gestational exposure; the findings were criticized, however, for a lack of concordance among twins (who presumably shared the gestational risk factor), and a follow-up study failed to replicate the findings [65,66].

Several other epidemiological surveys have considered early-life infections in the etiology of PD, but there are significant problems including recall and selection biases, selection of cohorts which may not yet have reached the mean onset age of PD, variations in diagnosis and reporting of the infection(s) throughout various geographical and historical periods, sub-clinical (and thus unidentified) infection, and latency; these studies have met with negative and even contradictory results, but they have investigated the important hypothesis that early life exposures can influence adulthood neurological disease [66–72]. These epidemiological studies, while thus far failing to identify a relevant infectious etiology, highlight some of the challenges in linking more recent experimental evidence for developmental environmental risk factors with human populations.

2.2. Experimental models with perinatal exposures

Experimentally studying the role of the post-natal environment in the etiology of PD has recently identified a series of chemical exposures that may hold implications for PD, and these studies serve as a foundation for considering the exposures that may be relevant in establishing PD as an example of FeBAD. Paraquat (PQ) and maneb (MB) are agricultural chemicals that have each been linked to nigrostriatal damage and the emergence of Parkinsonian symptoms, via epidemiological surveys, clinical case reports and/or animal models ([73–82]). In rodents, the combined exposure to PQ + MB causes selective and potentiated damage to the nigrostriatal system [10,74,83]. Based on the premise that PD could arise from events in early development followed by long-term and delayed consequences, neonatal mice were administered these compounds, alone or in combination on days 5–19, and then re-challenged when they were 6 months of age [17]. Locomotor activity, neurochemistry and DAergic cell counts suffered the greatest magnitude of adverse consequences in animals exposed to PQ + MB in both post-natal and adulthood periods, suggesting progressive effects and enhanced vulnera-

bility to known DAergic toxicants; furthermore, these effects were more pronounced in male mice, correlating to the known epidemiological predominance of PD in males [45].

In another recent study, a cohort of mice that was fed an iron-enriched diet was sacrificed at various points in life (ranging from 2 months to 2 years) after a challenge with the DAergic toxicant MPTP, and examined for alterations in the nigrostriatal system, including DA levels and DA neuron number [61]. By studying these mice across the lifespan, researchers discovered that the iron-enriched diet led to enhanced vulnerability to MPTP that only became apparent in advanced age, and that iron exposure in the neonatal period itself accounted for a significant reduction in DAergic neurons in very old mice. These results suggest silent toxicity with unmasking, latency, and progression, which are all very important features in establishing a developmental or gestational model of PD.

Organochlorine pesticides (such as heptachlor and dieldrin) are persistent environmental contaminants that have also been implicated in the etiology of PD [84], and evidence suggests that perinatal exposure may alter DAergic neuron phenotype to enhance vulnerability. Heptachlor was administered to young female mice for a period extending from 2 weeks before gestation, through gestation, and until weaning [85]. Biochemical characteristics of the nigrostriatal system of male progeny of these dams were studied in young adulthood, and persistent alterations in DAT and VMAT2 were detected. To extend the implications for perinatal exposure to organochlorine pesticides on persistent deleterious DAergic outcomes, a subsequent report describes effects of dieldrin, which was administered to gravid and lactating mice, with no immediate adverse outcomes on offspring [86]. When the pups reached 12 weeks of age, the researchers found alterations in a variety of markers for the DAergic system, including the proteins DAT, VMAT2, NURR1 and Pitx3; when MPTP was administered, they found greater adverse effects in animals perinatally exposed to dieldrin compared to controls. Importantly, the levels of dieldrin in the brain at 12 weeks were below the limit of detection, suggesting that changes in gene expression are persistent into adulthood even when the inciting toxic agent was no longer present.

2.3. Experimental models with prenatal insults

While these studies have identified a variety of environmental risk factors occurring during development that capture features of the multiple hit hypothesis, the gestational period is developmentally distinct from the neonatal period, and exposures may carry different consequences. Animal models have recently been described that specifically considered the role of gestational exposures in disrupting the nigrostriatal system, and each has implications for elaborating our understanding of the etiology of PD. Described below, one model involves a role for pro-inflammatory cytokines, which may relate to epidemiological studies of early-life infectious agents and intrauterine infections [87–89]. Also described below, another model relies on in utero exposure to the dithiocarbamate fungicide MB, which has previously been linked to Parkinsonism and DAergic dysfunction ([10,76,78,82,83,90–95]), combined with subsequent exposure to the herbicide PQ [12]. Another recent study started with the premise that there are known long-term alterations of DA neurochemistry following in utero cocaine exposure, and thus hypothesized that in a mouse model, an increased sensitivity to MPTP toxicity following gestational exposure would occur; the outcomes of in utero exposure were also compared to a chronic adult cocaine exposure paradigm [96]. While finding significant losses of DA neurons associated with the combination of cocaine and MPTP, the results suggest that in utero exposure did not confer any added risk compared to adulthood cocaine exposure, though it may indicate that pregnant cocaine users increase the risk of offspring to developing PD later in life. Key findings of these reports are summarized and compared in Fig. 2.

2.3.1. Bacteriotoxin LPS

Based on the findings that pro-inflammatory cytokines are increased in brain of PD patients, and that the bacteriotoxin lipopolysaccharide (LPS) is a direct DAergic toxin and inducer of cytokines, researchers hypothesized that administration of LPS to gravid rats would lead to adverse outcomes for the DAergic system of offspring [88]. After establishing a paradigm involving administration of 1 mg/kg LPS on gestational day 10.5, this study found that prenatal LPS decreased striatal DA concentration and decreased the number of DAergic neurons in both the SNpc and the ventral tegmental area (VTA) to a similar degree (∼25%) in 3-week old offspring of both genders (see Fig. 2A and C). Levels of cytokines were markedly elevated as well, even several weeks after the initial and only exposure, suggesting a persistence of effects. In a follow-up study, this group of researchers questioned whether the DAergic system already damaged by prenatal LPS exposure would be more vulnerable to the toxic effects of the known, potent toxicant 6-OHDA [89]. Using male offspring at a later time-point (3 months of age), they again observed that prenatal LPS reduced the number of DA neurons in the SNpc; when considered with results of the initial study, it appears that prenatal LPS reduces the "baseline" number of DA neurons in offspring, but that this baseline remains stable once it is established, even beyond 16 months of age (see Fig. 2, showing similar ∼20–30% reductions in SNpc DA neuron number across studies and across several ages of animals) [87–89]. Though animals exposed to LPS and then to 6-OHDA did indeed ultimately have lower numbers of DA neurons in the SNpc, there was a less-than-additive effect of LPS-prenatal + 6-OHDA in adulthood, suggesting that prenatal LPS did not confer any additional vulnerability to the DAergic system other than the initial reduction in the baseline (or "starting") number of neurons [89] (see Fig. 2B). A similar prenatal exposure paradigm was used in another experiment, but adulthood challenge was carried out in female offspring at 16 months of age with the pesticide rotenone [87], which has previously been used in animal models of PD [4]. Though rotenone treatment presented significant mortality and health risks ([43,87]), it did have adverse effects on the DAergic system (including a significant decrease in DA levels and decreased intensity of staining of tyrosine hydroxylase (TH), a key enzyme in DA biosynthesis and a marker for DAergic neurons), despite the finding rotenone did not directly lead to cell loss in these aged female rats. How-

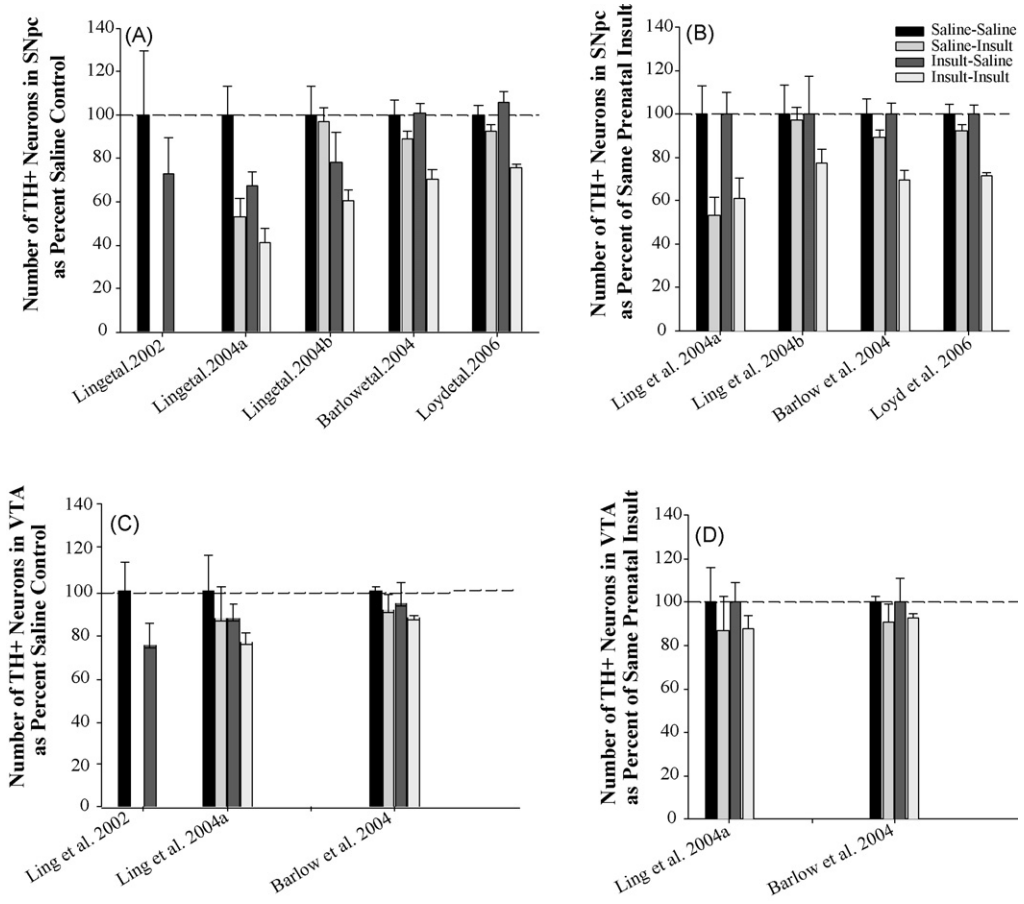

Fig. 2. Comparison of models of developmental toxicant exposures on brain dopamine cells. Several studies have considered the effects of prenatal insults (all include saline/vehicle controls; LPS exposure in Ling et al. [88], Ling et al. [89] and Ling et al. [87]; MB exposure in Barlow et al. [12]; cocaine exposure in Lloyd et al. [96]), with or without adulthood re-challenge (either saline/vehicle in all studies and either 6-OHDA [89], rotenone [87], PQ [12] or MPTP [96]), on the number of TH+ neurons in the substantia nigra (SNpc) and ventral tegmental area (VTA). To make appropriate comparisons between studies, each study was normalized to saline–saline controls (A and C), based on directly reported values (SNpc and VTA [88], SNpc [87,89]) or estimated based on published graphs (VTA [89], SNpc and VTA [12], SNpc [96]). To visualize interactions of prenatal insult with adulthood insult, values were also normalized to "prenatal condition" (saline/vehicle or insult; B and D). LPS exposure has consistently been reported to induce a decrease in TH+ neuron number in the SNpc by ~25–30% (A) [87–89], though effects on VTA have been reported as significant [88] and non-significant [89] (C). Neither MB nor cocaine prenatally caused a decrease in the baseline number of TH+ neurons in the SNpc (A) [12,96] and prenatal MB exposure did not affect the number of TH+ neurons in VTA (C) [12]. In the studies that considered effects of prenatal insult combined with adulthood insult, a clear interaction of the exposures can be observed for LPS + rotenone [87], MB + PQ [12], and cocaine + MPTP [96]; see (A and B), which normalizes to "saline–saline" and "insult-saline" to more easily visualize effects of adulthood insults); of these interactions, the effects were selective for the SNpc when reported [12]; see (D).

ever, against the background of prenatal LPS exposure, cell loss was significant in the SNpc, displaying an interaction of prenatal exposure and adulthood challenges, suggesting a role for age and multiple environmental hits. In the most recent study, 7-month old male rats exposed to LPS or vehicle prenatally were subjected to supra-nigral infusion of LPS, and sacrificed after 2 or 12 weeks [97]. As previously observed, prenatal LPS caused a decreased baseline number of DAergic neurons, though this prenatal background did not influence the magnitude of effect of the adulthood exposure, and there were no changes in TH expressing neurons over time to suggest a progressive decline, there was an accelerated microglial response to in the LPS–LPS group, compared to others.

2.3.2. *Maneb + paraquat*

Because the pesticides MB and PQ have been associated with PD via many lines of evidence, our laboratory examined whether prenatal exposures to these agents would cause permanent, progressive and/or cumulative adverse effects on offspring, and whether vulnerability to future toxicants would be increased following prenatal exposures [12]. Pregnant mice were treated with either saline, MB (1 mg/kg in saline, corresponding to 0.3% of the LD_{50}) or PQ (0.3 mg/kg in saline, corresponding to 1% of the LD_{50}) daily on days 10–17 of gestation. Pups were weaned at day 25, and when they reached 6 weeks of age, these adult animals entered into a re-challenge period in which they received either saline, MB (30 mg/kg) or PQ (5 mg/kg) for 8 consecutive days. (This resulted in the following experimental paradigm: three prenatal conditions (SAL, MB or PQ) × three adulthood re-challenge conditions (SAL, MB or PQ) × both genders.) Outcome measures included locomotor activity assessment, determination of striatal and cortical DA, DA metabolite, and serotonin concentrations, and stereological assessment of TH+ and TH− neurons in the SNpc and the VTA.

None of the prenatal conditions caused any adverse effect on gestation, parturition, litter survival or growth at any point, nor was there any morphological or behavioral teratogenicity associated with any prenatal exposure. The most significant finding associated with this study is observed in males exposed prenatally to MB and to PQ in adulthood. Despite a marginal increase in locomotor activity during the 8-day exposure regimen, 1 week following this re-challenge period, a dramatic decrease (95%) in activity was observed only in this treatment condition. This finding was supported by decreased levels of striatal DA, increased striatal DA turnover and selective reduction in TH+ neurons of the SNpc (while TH− neuron number and cell composition of the VTA was unchanged). These gender- and region-specific changes were related to a silent toxicity with unmasking, were persistent (as detected by cell loss, observable 1 week after exposure ended), and were due to a specific order-of-presentation of the pesticides (rather than just a combined effect).

Our study on the effects of prenatal MB exposure presents several key features necessary in a model of PD as an example of the FeBAD hypothesis, and introduces a large set of as-yet unanswered questions. Firstly, this model fits Reuhl's definition of silent toxicity [27], where a prenatal exposure caused no teratogenicity, though a large and specific interaction is observed when the unmasking challenge is introduced. Secondly, there is a latent period across which the silent toxicity was maintained. The characteristics of the nigrostriatal system during this latent period are unknown, though long-term alterations in DA-related mRNA levels and expression have been observed in at least one other developmental model of PD [86]. Unlike the LPS model, it is clear that prenatal MB did not lead to a reduction in the baseline number of DAergic neurons; mice exposed to prenatal MB entered adulthood with the same number of DAergic neurons as non-treated peers, though it is now hypothesized that these neurons exist in a state of altered potential that increases vulnerability to cell death, consistent with the model depicted in Fig. 1A [19,28]. Thirdly, there is a persistence and specificity of effects, where the adulthood insult (PQ) caused cell death specifically in DAergic neurons of the nigrostriatal system. This model also provides evidence for the multiple hit hypothesis, since adverse outcomes required two episodes of exposure (separated temporally), before there was a breach in the threshold level to cause dysfunction.

2.3.3. Comparison of models

Because DAergic cell death is the ultimate end-point in describing features of PD, and because this end-point has been reported in many of the relevant studies, Fig. 2 provides a way to visually compare the key findings of the studies that experimentally considered gestational environmental exposures on the DAergic systems, with or without a DAergic insult later-in-life [12,87–89,96]. Prenatal LPS has consistently caused a stable reduction (reported range 22–33%) in the number of TH+ neurons in the SNpc, as measured at 3 weeks [88], 3 months [89] or 16 months of age [87] (Fig. 2A), though effects on TH+ neurons in the VTA are equivocal (for example refs. [88,89]) (Fig. 2C); these stable reductions are consistent with the model depicted in Fig. 1B, where an acute hit during gestation causes an immediate drop in functionality of the system, with resumption of a course parallel to normal. Though it remains to be fully characterized, evidence with prenatal LPS and adulthood rotenone, suggest a model more similar to Fig. 1C, where acute hits cause immediate damage and a more vulnerable steady state. Prenatal MB [12] and prenatal cocaine [96] do not cause immediate reductions in the number of DAergic neurons (Fig. 2A), and though it is not fully characterized, it can be hypothesized that these toxicants establish a state of increased vulnerability (as detected by an interaction with adulthood challenges), suggesting a model more similar to Fig. 1A. When normalized to baseline values (the stable state that follows prenatal exposures), three of the models clearly show an interaction between prenatal insult and adulthood re-challenge (see Fig. 2B): LPS + rotenone [87], MB + PQ [12] and cocaine + MPTP [96]. Based on the observed interactions, these exposure paradigms each have features that suggest that they may serve as a model for environmental exposures, disrupted neurodevelopment and later neurodegeneration.

3. Studying the neurodevelopmental basis of neurodegeneration

3.1. Epidemiological challenges

Studying a role for the gestational environment in the etiology of PD, and thus making this disease a relevant example of the FeBAD hypothesis, presents many unique challenges, and characteristics of these challenges can in part be attributed to the foundations of FeBAD in two distinct but inter-related fields: epidemiology and developmental toxicology [18]. It is important to state that aside from the few studies herein reviewed, PD has almost exclusively been considered as a disease of aging, with few epidemiological studies considering the childhood or gestational environment. While animal models can help elucidate some of the potential environmental risk factors and their mechanisms of action in adulthood neurodegenerative conditions, there are several significant problems in considering these factors in human populations.

Because it is a disease associated with late-in-life onset, typical PD patients are less likely to have living parents or siblings to recount accurate environmental factors than are diseases with much earlier onset [98]. Most cohort studies for neurological diseases include individuals at age 65 or older, and a significant amount of recall bias and challenges are introduced by retrospective study of events occurring decades before the onset of symptoms [57]. It is very difficult to obtain accurate information about the fetal environment, especially if biomarkers are not available or are unreliable; prospective studies provide a solution, but long latency until disease emerges, the tracking of participants, the identification of exposures and consistent diagnosis all require decades of follow-up, and limitations are introduced by the type, frequency, and periodicity of data collection, so that, ultimately, these types of studies must include large numbers of subjects to assess multiple risk factors [22].

A possible reason for the lack of identification of specific environmental risk factors in PD is the focus on events and exposures that occur in adulthood, even though evidence is building

that the initial predisposing event occurs years before disease onset [68,69], and researchers have been calling for a focus on childhood environments to gain important insight into the etiology of PD for many years [53]. The long latency between gestational exposure and onset of disease presents another epidemiological challenge that is well-illustrated by the attempts to link gestational influenza with risk of PD [64,65]: when studying birth cohorts from a period of less than 60 years in the past, it is clear that individuals in those cohorts have not yet reached the average window for onset of PD, so there may be many "pre-patients" (individuals in the very long period of sub-maximal DAergic function before threshold is breached and symptoms become apparent; see Fig. 1) who were wrongly identified as normal controls. (Significantly, the issue of the pre-patient is also a major challenge in the study of "normal" aging, since individuals with a vulnerability to developing clinical symptoms of PD may be wrongly classified as normal controls, and thus, in human post-mortem studies, potentially introduce false data points that bias toward the hypothesis that DAergic function does exhibit a normal decline with age.)

3.2. Experimental challenges

In addition to these challenges presented from the "epidemiology side" of studying PD as it relates to the FeBAD hypothesis, there are specific challenges related to studying the "developmental toxicology side" as well. When silent toxicity is implicated, the chances of identifying a relevant environmental risk factor is even further diminished, since by definition, silent toxicity is unapparent without unmasking (unless some known "footprint" or biomarker could be detected without the unmasking challenge) [27]; even if a hypo-DAergic state *were* present in a child, for example, the child may easily evade clinical attention because, perhaps, s/he would simply be considered shy, introspective, and agreeably hypoactive [71]. Additionally, as highlighted by the currently presented reports, silent toxicity and unmasking challenges are fundamental to advancing our understanding of gestational environmental factors in PD. Studying toxicants as single agents does not adequately address the risks of subsequent disease [44]. Furthermore, as originally outlined by Reuhl [27], choosing relevant unmasking agents based on solid epidemiological and biological rationale is a challenge, since it requires a thorough knowledge of the target system and the pathophysiologic process, a solid understanding of toxicological principles especially as they relate to reproduction/gestation, development, and aging, the identification of known or likely risk factors/combinations based on epidemiological findings and public health realities, a significant investment of time and capital to establish relevant cohorts, exposure paradigms, and endpoints, and an expertise for appropriately interpreting results due to interacting toxic agents.

3.3. Features for a development animal model of PD

The process for experimentally investigating a role for the gestational environment in the etiology of PD should follow the same basic steps as other models of FeBAD: exposure to non-teratogenic doses/agents, prospective study across the lifespan for signs of dysfunction, characterization of tissue-specific gene expression over time, demonstration of a cause-effect relationship between exposure, gene expression, and incidence/severity of disease and determination of the mechanism of toxicity [18,19]. Combined with these features, we also suggest additional features for studying a role of the fetal environment and subsequent neurological disease, especially as it relates to PD; some of these features, as described below, have been achieved in the various developmental models of PD previously described. Though no model of gestational exposures satisfies all criteria yet, elaborating these features will provide a framework in which to the study of the role of the gestational environment on neurodevelopment and the subsequent risk for the neurodegeneration seen in PD.

3.3.1. Silent toxicity

First, as already proposed, a state of silent toxicity should be established and demonstrated, in which no adverse effects on gestation or parturition and no overt morphological or behavioral teratogenicity is observed [19,27]. Though the consistent finding that LPS is able to stably and persistently reduce the number of DAergic neurons from 3 weeks to 16 months of age [87–89], it is unclear whether this exposure can truly replicate features of silent toxicity. Even at low doses, LPS administration at mid-gestation is associated with severe adverse gestational outcomes in rodents; LPS induces behavioral abnormalities, decreased feeding, and early parturition in gravid animals [88], an increased resorption of fetuses, and a variety of malformations in offspring (for example refs. [99,100]). Furthermore, the reductions in TH+ neuron number observed at birth ([87–89]) are of similar magnitude (∼33%) to reductions that produce enormous behavioral disturbances in a mouse model of PD (∼30%) [12] (compare across studies in Fig. 2A) and which cause significant behavioral effects in rats [101]. In the absence of reports on initial behavioral outcomes in LPS-exposed offspring, it is unclear whether such an exposure would remain silent in comparison to non-exposed controls.

In contrast, the dose of MB used by our group is far below the teratogenic dose of this compound (which, in mouse, has shown limited or absent teratogenicity even at doses greater than 1 g/kg/day of gestation [102–104]), with no adverse effects on gestation, parturition, growth of offspring, or behavioral outcomes [12]. Though gestational cocaine exposure is associated with behavioral teratogenicity (see, for example ref. [105]), this model presents a different paradigm than either MB or LPS, since it is generally a self-administered "exposure" in the human population; thus, while it may not fit the criteria for silent toxicity, it does offer a unique advantage, epidemiologically, since children born during the cocaine epidemic of the 1970s to 1980s provide a human population (which will continue to age) in which to study the hypothesis that in utero cocaine exposure presents a risk factor for later developing PD [96].

3.3.2. Unmasking

Following silent toxicity, the second important feature for studying a role of the fetal environment on subsequent neuro-

logical disease is choosing a relevant unmasking agent, with evaluation of outcomes that appropriately detect selective damage to the target system. Toxicologically, MPTP, 6-OHDA, rotenone and PQ are all known to be selective DAergic neurotoxicants and have been extensively utilized in generating experimental systems (for review, see ref. [106]); though these agents are all powerful tools in experimentally studying PD, epidemiologically speaking (and discussed below), they are not all relevant to human populations. As it specifically relates to PD, the unmasking challenge should reproduce in the experimental animal several of the key features of PD; these would include behavioral changes (such as reductions in locomotor activity, as shown in the prenatal-MB–adulthood-PQ model), striatal DA depletion (as shown in the prenatal-LPS model and the MB + PQ model) and selective loss of DA neurons in substantia nigra (to date, shown only in the MB + PQ model; see Fig. 2).

3.3.3. Prospective study

A third and very important feature is the prospective study of experimental animals across the lifespan for markers of neurodegeneration (note that since normal aging itself is a controversial risk factor in PD and may present an endogenous "hit" (see Fig. 1), the prospective study should be conducted with and without the unmasking agent). Though to date no FeBAD model of PD has yet considered a cohort of animals as they age, two perinatal models (discussed above) have shown progressive neurodegeneration that was exacerbated by an environmental insult [17,61]. This type of cohort analysis, when studied with and without adulthood challenges, will provide a powerful tool in understanding the state of vulnerability, as well as the interaction of various environmental factors and aging. Fig. 1, as a hypothetical model, demonstrates that there are many questions regarding the emergence of PD and the relationship of events over the lifespan, and cohort analysis will help answer key components of this model, and potentially establish a link between gestational insults, adulthood environments, and the progressive neurodegeneration characteristic of PD.

3.3.4. Latent period

A fourth feature in studying PD as an example of the FeBAD hypothesis is the period of latency, during which a state of altered potential (or mutant steady state) is maintained [19,28]. This state represents a period of clinically absent symptoms during which the DAergic system is vulnerable and functionally impaired (Fig. 1). These states are suspected to be caused, developmentally, by epigenetic changes, meaning that gene–environment interactions change gene expression (without a change in DNA sequence) via mechanisms such as DNA methylation and DNA packaging around nucleosomes [107]; however, the specific mechanism(s) by which an otherwise innocuous environmental insult occurring in a developmental window of susceptibility can have long-lasting subtle consequences on gene expression is not yet known [108] Though none of the above models have yet fully characterized the state of vulnerability, the altered programming and subsequent functional changes induced by developmental toxicant exposures can be studied using a variety of omics technologies across the lifespan [18]. Gene expression profiling of human PD patients has identified several pathways in which genes are altered (for example ref. [109]), and many of these alterations correspond to those changes observed in animal models [110]; furthermore, examination of neurons in the SNpc and VTA has revealed anatomically differential gene expression, which may account for the preferential vulnerability to toxicants in some models of PD, and the regional selectivity of neurodegeneration in human cases of PD [111].

Though none of the developmental models of PD have yet reported broad analyses of gene expression, there have been some results that begin to characterize the state of altered potential. In the developmental model of pesticide exposures (see ref. [17]), our group found an up-regulation in D2-receptor like family genes and genes related to glutaminergic function, that was correlated to the in vivo findings (e.g. greatest changes with postnatal and adulthood exposures to PQ + MB) [46]. In the developmental model using dieldrin exposures, a persistent alteration in the ratio of DAT:VMAT2 (transporter proteins known to play a role in DA homeostasis) has been observed to alter susceptibility to later-in-life damage [86]. In beginning to characterize the state induced by prenatal LPS exposure, pro-inflammatory cytokines were found to be persistently elevated in striatum and midbrain [87–89]. Characterizing the state induced by prenatal toxicants may also provide insight into the ways the nigrostriatal system attempts to maintain homeostasis (and the ways in which homeostasis fails); a full understanding of this altered state may also help us understand the events occurring during the long period of latency and the extensive "pre-symptomatic" phase of PD, and may eventually lead to novel neuroprotective strategies to forestall, prevent or attenuate the progression of symptoms [14,47]. Additionally, the role of gender, genetic background and aging may also be clarified, as these factors are well-known modifiers of disease susceptibility.

A corollary of all of the features so far described is persistence of effect (of the inciting agent, of the unmasking agent, of progression and of latency and the state of altered potential). It is important to observe that the hits on the nigrostriatal system are not transient, but rather persist through each phase of the modeled illness: that silent toxicity induces an irreversible change in the system, that the mutant steady state is truly a stable (albeit vulnerable) system, that in addition to behavioral and neurochemical changes, the damage induced by the unmasking agent results in cell loss, and that progression (as a kinetic process distinct from stable persistence) results in increasing and cumulative damage across the lifespan. Furthermore, it is worth emphasizing that to relate these factors to a FeBAD model of PD, progressive and selective damage to the nigrostriatal system should be demonstrated. Though this "dopaminocentric" perspective on PD has recently been criticized as too narrow in scope (considering that features like protein inclusions, inflammation, oxidative stress markers, and peripheral and cognitive features are characteristic of idiopathic PD) [13], the clinical and pathological diagnosis of PD is defined by motor disturbances (i.e. tremor, bradykinesia/akinesia, rigidity) and nigrostriatal DA and DAergic-cell depletion; while they are correlated to PD and may help to establish mechanisms of the disease, the other

listed factors may be cause, consequence or independent of DA dysfunction and should not be relied upon to define a PD model.

While we currently hypothesize that the prenatal environment can induce disease susceptibility, PD is a neurodegenerative disorder characterized by progressive decline in DAergic function concurrent with an increasing severity of clinical symptoms (Fig. 1A). No model of prenatal exposures, with or without adulthood insult, has yet demonstrated progression (though other developmental models have provided evidence that it may occur following environmental hits [17,45,61]). The demonstration of progression will rely on the prospective study of cohorts of animals, observed at different points in the lifespan, with or without unmasking challenges. While studies of such factors as gene and protein expression, oxidative stress and inflammation may help characterize the system in the state of altered potential and the state of disease, the most relevant markers in modeling PD will remain motor function, neurochemical analysis and selective neuroanatomic lesions.

3.3.5. *Epidemiologic relevance*

While the factors thus far discussed have been based in developmental toxicology, the fifth and final factor we propose for establishing a model of gestational environmental factors in the etiology of PD is the selection of toxic agents that are epidemiologically relevant. As has been stated previously, cocaine exposure is a special circumstance that may prove to offer significant epidemiological advantages, since exposure is generally self-administered and historical context has provided large cohorts which may be studied as they age. MB is a dithiocarbamate (DTC) fungicide and is not a restricted use pesticide, and has seen increasing use over decades for a broad range of agricultural applications (on crops as varied as fruits, nuts, grains, seeds, vegetables, tobacco and ornamental plants); this compound can persist in the environment for weeks in soil and on food products, even after washing and extensive processing [82,112–119]. Residues from compounds like MB are among the most frequently found residues on agricultural commodities in Europe (see ref. [115]). Though MB is the DTC often used experimentally, it is but one of a larger set of DTC pesticides that share a similar mechanism of toxicity [95].

The pesticides rotenone and PQ are potent DAergic neurotoxicants (see ref. [106]) and theoretically present reasonable environmental risk factors in human populations, though only PQ (but not rotenone) has been epidemiologically linked to PD (for review, see ref. [6]). Nearly 1 million pounds of PQ were used on an extensive range of crops in 2003 in California alone, and over 1 million pounds of MB were used in the same year in the same geographical area, and these values reflect an increase in use of these chemicals from previous years [120]; exposure to the combination of these compounds is an environmental reality, and interestingly, incidence rates of PD in the US overlap areas of greatest MB and PQ use (see ref. [83]). A recent study of workplaces in Costa Rica found these two compounds to be among the most ubiquitous pesticides [121]. Furthermore, communities can be affected by drift of aerial applications or accidental spills [122,123]. In human populations, significant pesticides exposures do indeed occur during pregnancy with subsequent adverse effects on growth and neurodevelopment, and despite challenges in identifying these compounds, they are an epidemiologic reality (for recent examples, see refs. [124–131]). In sum, considering environmental realities and combinations, MB and PQ do represent relevant environmental agents to use in the establishment of a developmental model of PD.

In addition to its known toxicological effects on the induction of pro-inflammatory cytokines and resultant DAergic dysfunction, LPS was chosen for study in a model system based on a link to the clinical disease bacterial vaginosis (BV) [88]. BV is a common condition, accounting for more than a third of all vaginal infections [132]. A recent meta-analysis of obstetrical outcomes has confirmed significant adverse birth outcomes associated with BV, including increased risk of preterm delivery (especially if infection was early in pregnancy) and increased risk of spontaneous abortion [133]. BV has also been linked to low birth weight and is suspected to play a major role in many cases of infertility (via implantation inhibition and fetal loss at any stage of pregnancy); in addition LPS has known adverse effects on steroid hormone pathways [132], which may interact with other factors in the inflammatory process to cause adverse outcomes in pregnancy and in offspring. Though argued that BV can be a sub-clinical infection, the doses of LPS used in the recently proposed model of PD are large enough to cause adverse behavioral effects in the pregnant dam and pre-term delivery, suggesting that an infection of corresponding magnitude in a pregnant woman would not remain silent.

In addition, though BV is associated with low birth weight, an epidemiological survey considering this factor failed to associate it with risk for developing PD later-in-life [69]. Epidemiologically, because BV, birth weight, and pre-term delivery represent clinical outcomes that would be recorded as part of an individual's health history, the data should be available to further study this question retrospectively. While the LPS model will continue to provide insights into the mechanisms of DAergic dysfunction, until BV or LPS are linked to human cases of PD (via direct diagnosis of Gram-negative organism infection or by proxy measures such as chorioamnionitis, pre-term delivery or decreased birth weight), it is unclear whether this presents an epidemiologically relevant model in which to study the relationship of the FeBAD hypothesis to PD.

4. Conclusion

The future goals in establishing a role for the gestational environment on adverse outcomes of neurodevelopment, with subsequent vulnerability to the neurodegenerative disorder, PD, should focus on meeting the criteria in this review, as elaborated from previous reports [18,19,27]: gestational exposure causing silent toxicity, relevant unmasking techniques, prospective assessment across the lifespan, demonstration of progression (with or without unmasking events), characterization of the latency period (including gene expression and biochemical profiles) and the disease state, selective damage to the nigrostriatal DA system, studies to elucidate mechanisms of toxicity and the correlation to human populations via epidemiological findings. Recommendations for studying the neurodevelopmental origins

of neurodegenerative disease have also called for long-term prospective epidemiological studies to identify environmental factors, registries for people with PD to better track incidence rates, the study of unique disease clusters or environmental exposures, and improved techniques for toxicity assessments [48], which too often rely on the study of single acute agents without consideration of latent periods or the environmental reality of exposures and risk modifiers such as genetics, gender, nutritional status, comorbid disease, developmental stage or lifestyle factors [44]. There is a long history of hypotheses, and now compelling evidence, to suggest that the gestational environment can alter neurodevelopment, such that an individual is susceptible to developing a neurodegenerative disorder like PD later-in-life; interactions of prenatal environment, adulthood environment, gender, age and genetic background may also modify this risk. Studying this question in the context of experimental toxicology and human epidemiology will help define the relationship between the FeBAD hypothesis and PD, and will provide important insights into the etiology, progression and potential intervention strategies for PD.

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