Early-Life Toxic Insults and Onset of Sporadic Neurodegenerative Diseases-an Overview of Experimental Studies.

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Abstract

The developmental origin of health and disease hypothesis states that adverse fetal and early childhood exposures can predispose to obesity, cardiovascular, and neurodegenerative diseases (NDDs) in adult life. Early exposure to environmental chemicals interferes with developmental programming and induces subclinical alterations that may hesitate in pathophysiology and behavioral deficits at a later life stage. The mechanisms by which perinatal insults lead to altered programming and to disease later in life are still undefined. The long latency between exposure and onset of disease, the difficulty of reconstructing early exposures, and the wealth of factors which the individual is exposed to during the life course make extremely difficult to prove the developmental origin of NDDs in clinical and epidemiological studies. An overview of animal studies assessing the long-term effects of perinatal exposure to different chemicals (heavy metals and pesticides) supports the link between exposure and hallmarks of neurodegeneration at the adult stage. Furthermore, models of maternal immune activation show that brain inflammation in early life may enhance adult vulnerability to environmental toxins, thus supporting the multiple hit hypothesis for NDDs' etiology. The study of prospective animal cohorts may help to unraveling the complex pathophysiology of sporadic NDDs. In vivo models could be a powerful tool to clarify the mechanisms through which different kinds of insults predispose to cell loss in the adult age, to establish a cause-effect relationship between "omic" signatures and disease/dysfunction later in life, and to identify peripheral biomarkers of exposure, effects, and susceptibility, for translation to prospective epidemiological studies.

KEYWORDS:

Alzheimer's disease; Exposome; Heavy metals; Maternal immune activation; Neurodevelopment; Parkinson's disease; Pesticides

Tartaglione, Venerosi and Calamandrei, contribution to "Neurotoxin modelling of brain disorders"

EARLY LIFE TOXIC INSULTS AND ONSET OF SPORADIC NEURODEGENERATIVE DISEASES. AN OVERVIEW OF EXPERIMENTAL STUDIES

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Keywords

1. General Introduction

Since the publication in 1993 in *The Lancet* of the paper by Barker and co-workers (1993) indicating that fetal undernutrition was a major risk factor for cardiovascular diseases, a great body of experimental and epidemiological evidence has been accumulating, indicating that exposure to an unfavourable environment in early life is associated with a significantly increased risk of later disease, a phenomenon termed 'early life programming'. The Barker's hypothesis formed the basis for the developmental origins of health and disease (DOHaD) hypothesis that, further to fetal nutrition, considered a wider spectrum of adverse fetal and childhood exposures that can predispose to chronic diseases later in life. In such expanded view of the original Barker's hypothesis, not only malnutrition, but also the quality of the maternal diet, the intake of micronutrients, maternal smoking, and prenatal maternal stress may account for the relationship of the prenatal environment to adult disorders including cancer, metabolic, neuroendocrine, and neurodegenerative and cognitive diseases in adult life (Gillman 2005; Hanson 2013; Tarantal and Berglund 2014).

Knowledge about the mechanism(s) by which fetal insults lead to altered programming and to disease later in life is still scarce and, as discussed in the following paragraphs, it is mainly derived from experimental studies. The long latency between exposure and disease and the lack of a clear pathological phenotype before disease onset make extremely difficult to assess the underlying mechanisms in clinical studies. Lahiri and co-workers (2009) suggested that environmental factors play a role in chronic disease etiology by inducing latent epigenetic changes. These authors described the association of early environment with disease onset especially with respect to Alzheimer's disease (AD), proposing a Latent Early-life Associated Regulation' (LEARn) model that considers environmental exposures as 'hits'. According to this model, all neurodegenerative disorders come under the category of a 'n' hit latent model, where early life exposure leads to epigenetic perturbations in the genes, which remain silent until a second hit triggers the development of disease. The multiple hit hypothesis appears as particularly suited to explain the etiology of complex neuropsychiatric and neurologic diseases that share a highly sporadic nature characterized by genetic and environmental risk factors. It also explains the relatively long latency between exposure and disease, as subclinical deficits occurring during specific windows of developmental plasticity do not become manifest without a second hit occurring later in life (Heindel et al. 2015).

The DOaHD models, to date, have focused mainly on skewed nutritional states and/or low birth weight (Morley 2006), while the role played by exposures to environmental agents, such as water and air pollution and chemicals in food, either alone or in combination, has only recently come to attention. This is somewhat surprising since there is vast literature on the developmental

neurotoxicity that indicates an inverse association between chemical exposure and child neurode-velopment (Bellinger 2014 for an updated review). Extensive evidence also shows that some environmental agents alter developmental programming via changes in gene expression or imprinting that do not result in malformations but in functional deficits that become apparent later in life. However, much less attention has been posed to the role that early exposure to environmental toxins may play in predisposing to neurodegenerative and neuropsychiatric diseases in adult or even old age. This can be explained by several factors: the methodological constraints related to the difficulty of establishing cause-effect relationships between early exposure and disease manifestation (discussed in section 2.4), the lack of reliable biomarkers that can predict increased risk in early life stages, together with the still diffused idea that neurodegenerative diseases (NNDs) are linked to pathological brain aging.

In the present review, we will describe the general frame of the developmental origin of NDDs, and the challenges posed to experimental and epidemiological research investigating the role of environmental pollutants as risk factors. We will then present an overview of animal studies that have assessed the delayed effects of either single or multiple perinatal toxic insults, evidencing neurobehavioral alterations resembling the major hallmarks of human NDDs.

2. Developmental neurotoxicity and neurodegenerative diseases: a common playground?

2.1 Introduction

The human nervous system develops over a very long period extending from the embryonic period through puberty. About two weeks after conception, the neural plate folds to form the neural tube, and cells formed during this period of rapid proliferation migrate to a different position where they differentiate into neurons and neuroglia. In the human fetus, cell migration is nearly complete in the neocortex and in most of the brain by the sixth month of gestation (Gupta et al. 2005). During the remainder of intrauterine development, neurons differentiate and connect with each other. The period of rapid synaptogenesis or the so-called brain growth spurt (in humans occurring between the last three months of pregnancy and the 2nd postnatal year) is considered one of the most important processes that take place during brain development (Garner et al. 2002). This process is crucial not only in neurodevelopment but also plays a vital role in synaptic plasticity, learning, memory, and adaptation throughout life. Without this process, no complex brain network can be established, as synapse is the fundamental unit of connectivity and communication between neurons (Tau and Peterson 2010). During the brain growth spurt substantially more connections are formed than those that will be eventually retained as many of them will be gradually discarded by apoptotic

mechanisms. The process of synapse elimination is a normal part of development (Tau and Peterson 2010; Shonkoff 2000) and both endogenous (neurotrophic factors, synthesis and release of neurotransmitters, hormones) and environmental factors (i.e. sensorial inputs) concur to influence the fine matchup between pre- and postsynaptic neurons. Altogether, brain development is made up of several interactive and temporally overlapping stages. A plethora of mechanisms regulates each of these sequential steps, and some of the major signaling cascades and trophic factors in the developing brain play a role in the aging or neurodegenerating brain. In a recent review, Kovacs and co-workers (2014) highlighted the commonalities between neuronal loss and neuronal development. These authors performed an overview of NNDs according to major proteins deposited (i.e. β−amyloid and tau protein); they observed that genes that are mutated in neurodegenerative diseases code for protein that are highly expressed throughout neural development. Characterization of the mechanisms underlying hippocampal neurogenesis in the fetal and adult brain point to the involvement of the same gene products, namely of proteins that seem to drive either synaptogenesis or synaptic dysfunction depending on the age factor. This is the case of reelin, presenilins (PS), Notch-1 and brain-derived neurotrophic factor (BDNF). Furthermore, proteins representing hallmarks of NDDs might also play a crucial physiological role during brain development and adult neurogenesis. Specifically, the amyloid precursor protein (APP) significantly contributes to neurite outgrowth and hippocampal development (Billnitzer et al. 2013), α-synuclein has a key role in synaptic vesicle recycling and is expressed in normal fetal brain, phosphorylation of the tau protein takes place during fetal development (Goedert et al. 1993). Altogether, though the majority of data originates from animal model research and still awaits confirmation in humans, there is substantial support to the view that pathological changes observed in NDDs could be in part the result of aberrant neuroplasticity mechanisms settled during the fetal life (Bugiani 2011). In this light, the study of the toxicants' effects on brain development is possibly crucial for unravelling the complex NDDs etiology.

2.2 Brain plasticity and vulnerability

The remarkable plasticity of the brain during the fetal and neonatal periods is a double-edged sword as it renders this organ extremely responsive to environmental factors. On one hand, early environmental inputs can interact with genetic factors to promote neural circuit and functions development (Sale et al. 2014); on the other hand, early negative conditions (i.e. stress, toxic insults) can act in the opposite direction, increasing the chances to develop neurodevelopmental disorders or diseases in adulthood. Diverse environmental stressors - chemical pollutants, drugs, nutritional factors, maternal infection, stress, deprivation - may interfere with typical brain developmental

trajectories. In order to cope with insults perturbing prenatal environment, the fetus develops compensatory strategies that could be adaptive or disruptive, resulting in interindividual variability in the total neuronal and glial burden and functional reserve.

Exposure to toxicants at any point during brain development may result in aberrant neural structure or function. The end points affected may vary depending on the timing and duration of exposure. Toxic disruption of early maturational events are more likely to result in neural tube defects or major malformation (i.e. anencephaly, spina bifida), while toxic interference with later developmental events results more commonly in cytoarchitectural and molecular alterations that might be expressed as behavioral dysfunction. Specifically, damage or destruction of neurons by chemical compounds when they are in the process of synapses formation, integration and formation of neural networks will derange the organization and function of these networks, thereby setting the stage for subsequent impairment of learning and memory (Kovacs et al. 2014).

It was not until the early 1990s that significant evidence emerged showing that low-dose exposures to metals such as methylmercury and lead, though not producing teratogenic effects evident at birth could result in later behavioral dysfunctions in children (Gilbert et al. 1995; Banks et al. 1997). Similar results were later identified for the perinatal exposure to other environmental contaminants such as pesticides (Eskenazi et al. 2007; Perera et al. 2009), polychlorobiphenyls (Winneke 2001), polybrominated diphenyl ethers (Herbstman et al. 2010), and phthalates (Whyatt et al. 2012). These observations were consistent with the emergent DOHaD concept, and shifted the emphasis of developmental toxicology from the study of teratogenic effects to functional changes occurring during development and becoming manifest only after a latent period (Bellinger 2009; Miller 1986). Mechanistic studies carried out in animals and in *in vitro* models point to multiple pathways and targets of toxicity for several established neurotoxicants, depending on the dosage or time window of exposure (Neal et al., 2011; Roy et al., 2004; Slotkin and Seidler 2009). A reconsideration of the targets of toxicity of the major environmental pollutants in the developing brain could possibly reveal significant commonalities between pathophysiology of neurodevelopmental disorders and the cognitive decline associated to NDDs (see the case of developmental lead exposure, section 3.2.1).

2.3 The multiple hit hypothesis for neurodegenerative diseases

Because the most of cases of NDDs onset late in life, they have been traditionally considered as linked to aging, but recent evidence suggests that the aging process is less important than was thought in the past. According to the LEARn model, all sporadic NNDs are characterized by ´n´ hit latent model. Their onset in adulthood may be the consequence of alteration in gene regula-

tion induced by early life exposures that, after a silent period, lead to disease. Under this perspective, the old age may be the point in time where any change in developmental programming produced by multiple hits interacts sufficiently to cause overt disease (Logroscino 2005). Under LEARn, early life stressors modify potential expression levels of disorder- associated genes in a latent manner. Latent changes in these genes are maintained by epigenetic mechanisms such as DNA methylation, DNA oxidation and chromatin reorganization. Alterations in gene expression lead to altered protein synthesis, and eventually to altered numbers and /or location of cells.

The multiple hit hypothesis firstly advanced to explain the etiology of PD (Calne and Langston 1985) might be applied to other major NNDs. In this light, the environmental insult could act via a one hit or even two/three hit scenario, where the fetal insult may be the first event. Fetal exposure to a toxicant would lead by itself to disease later in life, as the neurotoxicant may induce subclinical injury to neural or glial cells, which leads to progressive loss of function. The developing organism initially compensates for the mild deficit, but the functional reserve and plasticity of the brain would be overcome with time, and loss of function (including behavioral deficits) would appear (Rehul 1991). A mechanistic hypothesis proposed that early exposures to neurotoxic chemicals reduce the number of neurons in critical areas of the brain, setting in motion a self-perpetuating process of neurodegeneration (Langston et al. 1999). In some cases, the fetal exposure needs to be combined with a neonatal exposure either to the same or different compound or to an adult exposure to cause the pathophysiology. Thus, the initial deficit would only be unmasked by subsequent exogenous influences (stress, disease, additional chemical exposure) or by the natural aging process (Costa et al. 2004).

2.4 Environmental chemicals and neurodegenerative diseases: limits and constraints of epidemiological studies and the need of experimental models

A role for environmental toxicants in increasing risk of neurodegenerative diseases, such as PD and AD has been advanced based on case-control retrospective studies (Santibanez et al. 2007; Van Maele Fabry et al. 2012). Specifically, AD and PD have been associated to metals and pesticide occupational exposure, but data are far from being conclusive (Hertzman et al. 1990; Liou et al. 1997).

Testing the hypothesis of the neurodevelopmental basis of NNDs in human population presents significant methodological problems. One of the most significant challenges is the issue of silent toxicity, as the developing nervous system is endowed with marked ability to compensate for insults. At variance from neurodevelopmental disorders, exposures may have occurred many decades before the diagnosis of AD or PD; thus the assessment of chemical exposures is almost impos-

sible to reconstruct in retrospective studies. In addition, the lack of reliable biomarkers makes difficult to obtain information on the fetal environment of an adult individual. Prospective studies linking fetal exposure to adult diseases requires decades of follow-up, and may be confounded by multiple factors (i.e. co-exposure, life style, genetic vulnerability) modulating the risk of the original environmental insult. The methodological constraints widely recognized in neurodevelopmental toxicity studies, namely dose-response estimation, individual dimension of the exposure history and robustness of the clinical outcome (De Felice et al., 2015), are amplified by the "time" factor in studies on the developmental origin of NNDs. Of the 112 existing EU human cohorts established for studying NDDs, only five of them have followed people continuously from birth into adulthood; though many factors are currently under study (i.e. gene background, nutrition, life style) the issue of early exposure history is scarcely considered (JPND Action Group, 2013).

Overall, the epidemiological and clinical data collected so far, while supporting the hypothesis of a contribution of environmental factors to NNDs, highlight the enormous difficulty of establishing causative links between each of these factors and the health outcome. The simultaneous exposure of an individual to multiple risk factors, which may interact in an additive, synergistic, or even antagonistic way along the life course remains to be explained and defined. Recently, the exposomic approach, considering all the endogenous and environmental factors to which an individual is exposed during the life span, has suggested a procedural framework to understand the fetal origin of adult diseases. In the exposome framework, the complex origin of human chronic diseases can be unfolded by means of several omic signatures possibly describing exposure, effects, individual vulnerability and their dynamic interplay (Wild 2012). However, peripheral biomarkers of exposure so far available for many environmental chemicals are indeed poor predictors of effects on the brain (Stangle et al. 2004); to complicate the picture further, in the case of late onset neurologic diseases biomarkers of effect should be able to capture early biological changes and predict later health effects.

In this framework, studies with laboratory rodents allow evaluation of important parameters, such as dose-response relationships, critical periods of susceptibility, and the relative contribution of genetic, epigenetic and environmental factors. So far, the use of animal models has been of paramount importance to investigate the mechanisms by which chemicals influence brain development in humans (Vorhees 1986). In particular, animal models are a powerful tool to test what combination of adverse environmental factors produce significant disruption of neurobehavioral development that lead to clinically relevant outcome in later stages. Notably, this kind of information can only be achieved by testing the living organism, as the face validity of an animal model of NDD consists primarily in a robust behavioral phenotype that can be associated to

the appearance of neurodegenerative features (Crawley 2012).

3. Assessing the role of environmental exposures in neurodegenerative diseases' etiology: an overview of the in vivo models

3.1 Introduction

The role of environmental chemicals and their mechanisms of action in the etiology of sporadic AD and PD have been extensively discussed in the recent review by Chin-Chan and co-workers (2015). Based on epidemiological and experimental evidence, the authors identified neurotoxic metals, such as lead (Pb), mercury (Hg), aluminum (Al), cadmium (Cd), manganese (Mn) and arsenic (As), as well as some classes of pesticides as significant environmental risk factors. As mentioned above, most of studies suggesting association between these chemical agents and idiopathic AD or PD refer to occupational exposure (Santibanez et al. 2007; Firestone et al. 2010). Many are case-control studies (Parron et al. 2011) and very few population studies considered internal exposure to a specific compound in relation to disease occurrence (Richardson et al. 2014; Kim et al. 2015). The mechanisms by which chemicals with very different modes of action might promote neurodegeneration are still undetermined. For example, the pesticide class includes more than 1000 active ingredients including insecticides, herbicides and fungicides. Among those possibly implicated in AD or PD etiology, the organophosphorus compounds such as carbamates and organophosphate (OP) are potent inhibitors of acetylcholinesterase (AChE) and could affect APP processing (Giacobini et al. 1996); organochlorines (OC) can impair the functioning of the mitochondrial system and produce free radicals increasing oxidative stress and promoting cell death; bipyridyles such as paraquat (PQ) can generate free radicals that are able to cross the blood-brain barrier and damage neurons (Kumar et al. 2015).

In their extensive review, Chin-Chan et al. (2015) described several experimental studies (*in vitro* and *in vivo*) that have focused on metabolic pathways relevant for AD and PD pathogenesis. It is worth noting that in the large majority of the *in vivo* studies reviewed, rodents or non-human primates were exposed to chemicals at the adult stage. The metabolism of APP, phosphorylation of tau protein, aggregation of α-synuclein, production of proinflammatory cytokines and reactive oxygen species generation appear as common targets of metals and pesticides' neurotoxicity following adult exposure. Furthermore, transgenic (TG) models carrying mutation in candidate genes supports the hypothesis that both genetic vulnerability and environmental neurotoxicants converge on neuroinflammation as an outcome linked or leading to neurodegeneration. It is worth noting that the very earliest neuronal and pathological changes characteristic of AD suggest oxidative stress as

a very early contributor to the disease (Nunomura et al. 2001) and oxidative stress appears to play a major role in degeneration of DA neurons in PD (Dias et al. 2013).

In the following sections, we will present the more robust experimental studies including fetal and/or neonatal exposure to known environmental neurotoxicants, which support the link between chemical exposure and hallmarks of neurodegeneration at the adult stage. In many of the studies the significant alteration in one or more of these hallmarks was also paralleled by motor and/or cognitive disturbances, reproducing in the rodent model the clinical behavioral traits of AD and PD affected individuals in the initial stages of the disease. We chose to include in this overview also rodent studies exploring the delayed neurobehavioral effects of maternal infection. The paradigm of maternal infection models a condition of early immune activation and consequent exposure of the fetal brain to pro-inflammatory cytokines: this can affect the development of brain circuitries leading to neurobehavioral dysfunctions in the progeny (Knuesel et al. 2014). As shown below, studies combining maternal infection with a subsequent toxic insults support the validity of the multiple hit hypothesis, as an unfavorable fetal environment increases later vulnerability to neurotoxicants. Tables 3.a, 3.b and 3.c illustrate synthetically the experimental studies discussed in this chapter.

3.2 Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide. The main pathological hallmarks of AD are beta-amyloid plaques (Aβ) and neurofibrillary tangles (NTFs) composed of hyper phosphorylated tau protein (p-tau), which spread through the cortex in a predictable pattern as Alzheimer's disease progresses causing nerve cell degeneration and cognitive impairment (Selkoe and Schenk 2003). Early onset familial AD is rare and is characterized by autosomal dominant heritability, due to mutations in the genes for APP, presenilin-1 (PSEN1) and presenilin-2 (PSEN2). The prevalent form of AD typically develops after 60 years of age; the etiology of Late-Onset sporadic AD (LOAD) has not yet been resolved, but it has been suggested that it could result from a multifactorial process involving both genetic and environmental factors (Reitz et al. 2011). Diseases presenting increased inflammatory processes such as hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, obesity, and diabetes are indeed risk factors for AD (Blennow et al., 2006), thus inflammation is suggested to have a causal role also in LOAD pathogenesis. Several candidate risk genes have been identified for LOAD: they mainly involve proteins implicated in critical pathogenic pathways, namely Aβ synthesis and processing, oxidative stress and inflammation/apoptosis (Ballard et al. 2011).

Adverse environmental factors might further increase the risk impose by genetic make-up, by acting on cell pathways implicated in AD. Neurotoxic metals such as Pb, Hg, Al, Cd and As, as well as some pesticides and metal-based nanoparticles have been involved in AD due to their ability to increase Aβ peptide and the phosphorylation of tau protein, causing senile/amyloid plaques and (NFTs) characteristic of AD, and to enhance oxidative stress and inflammatory responses (Casado et al. 2008). In spite of this consistent evidence, studies considering environmental exposure have been underrepresented in the AD literature, possibly due to the challenges of retrospective exposure assessment in older adults (Bakulski 2012).

3.2.1 Heavy metals and AD

Lead

Among heavy metals, a large body of clinical data supports an association between occupational Pb exposure and cognitive decline with aging as higher levels of Pb in blood and/or bone were accompanied by poor cognitive performance in different cognitive tests (Nordberg et al. 2000; Wright et al. 2003; Weisskopf et al. 2004). Pb is a well-known neurotoxicant in children. Even at relatively low and subclinical levels, epidemiologic studies demonstrate prenatal and childhood Pb exposure affects IQ and behavior up to young adulthood (Lanphear et al. 2005; McFarlane et al. 2013). Recent studies using both *in vivo* models and primary neuronal cultures indicate that Pb exposure during pregnancy may negatively modify important neuronal pathways implicated in synaptic plasticity, learning and memory (Neal and Guilarte 2010). In this framework, the potential link between developmental exposure to Pb and increased risk of developing AD later in life has been thoroughly investigated in experimental studies with rodents and primates.

A line of research investigated the link between perinatal Pb exposure, alteration of APP metabolism and cognitive impairment at later life stages. Basha and coworkers (2005) exposed rats to Pb from birth through postnatal day (PND) 20 by systemic administration and monitored the lifetime expression of the APP gene, finding overexpression of APP levels and Aβ aggregation 20 months after exposure to Pb had ceased. These results have been replicated on monkeys, exposed to Pb from birth until the end of the first year of age (Wu et al., 2008). The expression of AD-related genes (APP, β-site APP cleaving enzyme 1 or BACE1), as well as their transcriptional regulator (Sp1) were elevated in aged (23-year old) monkeys following early exposure to Pb. Furthermore, levels of amyloid plaques increased in the frontal cortex. These latent effects were paralleled by a decrease in DNA methyltransferase activity and higher levels of oxidative damage to DNA, indicating that epigenetic mechanisms elicited by Pb in early life influenced the expression of AD-related genes and promoted DNA damage and pathogenesis.

Bihaqi et al. (2014) evaluated the impact of Pb exposure at different life times (during either development or adulthood) on cognitive functions and biochemical pathways associated with AD in mice. This study showed that only mice early exposed to Pb, namely during the first 20 days of postnatal life through the mother's milk, showed cognitive impairment at 18 months of age, which further progressed by 24 months. In Pb-exposed rats, cognitive impairment was accompanied by up-regulation of AD biomarkers (APP, Aβ and BACE1) and overabundance of p-tau. Long-term effects of developmental exposure to Pb has been evaluated also in relation to tau pathology in non human primates (Bihaqi & Zawia, 2013). In this study, female monkeys were exposed to Pb (1.5 mg/kg/day) from birth until the end of the first year of life and then they were examined at 23 years of age. Results showed that Pb exposure elevated the mRNA and protein levels of tau as well as its transcriptional regulators and favoured abundant tau phosphorylation in the frontal cortex of aged primates. This study suggests some evidence that Pb during development triggers neurodegenerative processes that have much in common with those implicated in human AD.

Due the role of developmental exposure of Pb in increase of Aβ, which is known to generate reactive oxygen species in the aging brain, Bolin et al. (2006) investigated the hypothesis that early exposure to Pb, in specific developmental window, determines the outcome of oxidative damage late in life. Rat pups were exposed to Pb at different ages (from PND 1 to 20 or from 18 to 20 months), showing that the level of 8-hydroxy-2′-deoxyguanosine (oxo8dG), one of the major products of DNA oxidation and a reliable marker of oxidative stress was elevated 20 months after exposure to Pb had ceased. The effect of Pb on oxo8 dG levels did not occur if animals were exposed to Pb only in old age. These increases in DNA damage occurred in the absence of any Pb-induced changes in general anti-oxidant capacity in the cerebral cortex.

The existence of a critical phase of sensitivity to Pb effects was also supported by other studies. Dosunmu et al. (2012) assessed global gene expression patterns in mice, developmentally exposed to Pb (0.2%) from gestational day (GD) 13 until PND 20. The selected time points of PND 20 and 700 represented early and late time points of the animal for microarray analysis, where genes that were down- and up-regulated were identified, clustered and analyzed for their relationship to DNA methylation. Prior exposure to Pb revealed a repression of genes related to the immune response, metal-binding, metabolism and transcription/transduction coupling. More in detail, the authors identified 150 genes for a transcriptional profile of normal aging and environmentally perturbed aging. In normal aging, those genes are up regulated, but prior developmental Pb exposure caused a strong repression of these genes. DNA methylation appears to play an important role in the downregulation of these genes suggesting that early life exposure to Pb interferes with the methylation pattern of genes and has an impact on the organism's capacity to

respond in old age. These findings would support the LEARn model of NDDs, as the toxic effects of Pb is maintained through the life span in the form of latent epigenetic changes.

Recently Ashok and coworkers (2015) treated rats with a mixture of As+Cd+Pb at environmentally relevant concentration, from GD 5 to 6 months of age. They identified dose-dependent increase of Aβ in frontal cortex and hippocampus as early as the post-weaning age. The metal mixture activated the pro-amyloidogenic pathway, mediated by increase in APP, and subsequent BACE1 and PS-mediated APP processing. Investigating the mechanism of Aβ-induction revealed an augmentation in oxidative stress-dependent neuroinflammation that stimulated APP expression. When the authors examined the effects of individual metals and binary mixtures in comparison with the tertiary mixtures they found that Pb triggered maximum induction of Aβ, whereas individual As or Cd had a relatively non-significant effect on Aβ despite enhanced APP. Together, these data demonstrate that exposure to As+Cd+Pb induces earlier manifestation of AD-like pathology that is synergistic, and oxidative stress and inflammation dependent. These data open the way to further experimental research modeling real exposure scenarios to mixtures of chemicals.

Developmental origin of Pb-induced neurodegenerative disease has been investigated also in Zebra fish (*Dania rerio*), an aquatic vertebrate model system, which presents a high sequence homology (i.e., 70%) with the human genome (Howe et al. 2013). Zebrafish are increasingly used to understand the mechanisms of developmental Pb neurotoxicity through evaluation of changes in gene expression and neurobehavioral alterations (Lee and Freeman 2014). Data collected in this model generally supported the hypothesis that developmental exposure to Pb exhibits latent effects on the expression of genes that are involved in neurodegenerative processes during old age.

3.2.2 Pesticides and AD

Several epidemiological studies have suggested the association between pesticides and impairment of cognitive functions and AD-like dementia, but the underlying mechanisms have been poorly explored in experimental models (Baldi et al. 2011; Hayden et al. 2010). Among the class of pesticides with neurotoxic activity, OPs are the subject of increasing concern. OPs make up approximately 50% of all insecticides used in the world (Colborn 2006), and are intensively investigated for their suspected developmental neurotoxicity (Rosas and Eskenazi, 2008). These compounds, largely used in agriculture, as well as in the home and garden, for pest control, exert their acute neurotoxic effects through AChE inhibition and consequent cholinergic stimulation. However, increasing evidence indicates that, as other environmental chemicals, OPs exert developmental toxicity at low doses with mechanisms different from those observed at higher doses, including modulation of several family of genes involved in brain development (Moreira et al.

2010). In vitro studies show that OP compounds impair mitochondrial bioenergetics and induce ROS generation (Wani et al. 2014). In particular, as concern chlorpyrifos (CPF), the most widely applied compound in the OP class in the USA, it interferes with brain and behavior development through a variety of cellular and molecular mechanisms that appears as independent from inhibition of AChE (Androutsopolous et al. 2013; Eaton et al. 2008; Venerosi et al. 2012).

Notwithstanding the recognized effect of this OP insecticide on oxidative stress end points (Cole et al 2011), developmental exposure to these compounds has never been considered under the perspective of neurodegenerative disease etiology. However, three recent studies using a genetic model of vulnerability to AD, the APP TG mice (Tg2576 mice) evidenced a potential increased susceptibility to both OP insecticides and bypiridyl herbicide paraquat (PQ). Specifically, CPF caused a significant increase in Aβ levels in the cortex and hippocampus, as well as increased memory loss and reduced motor activity in Tg2576 mice 6 months after a single subcutaneous administration (Salazar et al. 2011), an effect that has not been confirmed by Peris-Sampedro et al. (2014). Another study showed that treatment of wild type and Tg2576 mice with PQ (10 mg/Kg/twice a week/3 weeks) produced a significant increase in Aβ levels in transgenic mice, associated with mitochondrial oxidative damage in cerebral cortex and impairment of learning and memory. Interestingly, the overexpression of peroxiredoxin 3, a mitochondrial antioxidant defence enzyme produced an improvement in cognitive functions and a significant reduction in Aβ levels in Tg2576 exposed to PQ (Chen et al. 2012a), suggesting that pro-oxidant xenobiotics like PQ can contribute to AD.

3.2.3 Maternal infection and AD

As illustrated previously, experimental and clinical data support the inflammation hypothesis of AD etiology. It suggests that neuroinflammatory response triggers and follows the Aβ increase in the AD brain, and significantly interferes with memory processes (Heneka et al. 2015; Heppner et al. 2015). The administration of the bacterial endotoxin lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (polyI:C) to pregnant rodents were among the first animal models examining the long-term consequences of prenatal immune challenge on adult brain functioning (Meyer 2014). In experimental studies, LPS is used to increase pro-inflammatory cytokines in the pregnant female's organism, mimicking an infective state that can induce abnormalities in the fetus neurodevelopment (Knuesel et al., 2014).

Chen and coworkers (2011) injected female mice with LPS (50 mg/kg) daily during late pregnancy (from GD 15 to GD 17). The offspring exposed prenatally to LPS, although showing normal development of sensorimotor and cognitive functions, had an accelerated age-related

impairment of both spatial and non-spatial learning and memory in middle age. In a subsequent study by Krstic et al. (2012) female mice received a single injection of (PolyI:C) (5mg/kg) at GD 17. Adult and aged offspring showed cognitive decline and AD-like neuropathology such as increase of APP protein and its proteolytic fragments, altered tau phosphorylation, in parallel with impairments in working memory. A second immune challenge in adulthood exacerbated this pathological phenotype, with appearance of Aβ-like plaque deposition. In this same study, transgenic AD mice (3xTg), overexpressing the human variants of AD-relevant genes, were challenged with PolyI:C at the pre-plaque stage of 4 months, and showed accumulation of APP and increase of Aβ deposits resembling the morphology of Aβ plaques in human patients with AD. Taken together, these results indicated that systemic immune challenges are able to promote AD-like neuropathology in both wild-type (PolyI:C exposure at GD17) and genetically (3xTg-AD) predisposed animals, in agreement with both causative and exacerbating role of systemic immune challenges on AD development.

3.3 Parkinson's disease (PD)

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD, characterized by depletion of dopaminergic cell bodies in the substantia nigra *pars compacta* (SN*pc*) with subsequent loss of dopamine (DA) in the nigrostriatal system, and intracytoplasmic inclusions termed as Lewy bodies, containing aggregates of the protein α-synuclein as well as other substances (Barlow et al. 2007). Studies on familial PD show an autosomic pattern, either dominant or recessive, identifying some genetic mutations and chromosal loci responsible for this form. Several loci (PARK1-15) and genes have been linked to familial forms of the disease. The majority of PD cases are however, sporadic "idiopathic" forms and the recent application of genome-wide screening revealed almost 20 genes implicated in mitochondrial function, in detoxification and/or protection against oxidative stress that might contribute to disease risk. It is striking that none of the genes implicated to date is specifically expressed in DA neurons. Instead, many of the implicated genes have rather general neuronal or cellular functions in and outside of the brain, suggesting that DA neurons are more susceptible to stress than other neurons (Lees et al., 2009; Westerlund et al., 2010).

The cause of sporadic PD is unknown, with uncertainty about the role of environmental toxins and genetic factors. The environmental hypothesis posits that PD-related neurodegeneration results from exposure to a dopaminergic neurotoxin. The finding that people intoxicated with the chemical 1-methyl-4pheny 1-1, 2, 3, 6-tetrahydropyridine (MPTP) developed a syndrome nearly identical to PD (Langston et al. 1983) prompted the interest in environmental causes of PD (Tanner

et al. 2014). The herbicide PQ is structurally similar to 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of MPTP. Similarly to MPP+, the insecticide rotenone is also a mitochondrial poison present in the environment. Yet, there are no convincing data to implicate any specific toxin as a cause of sporadic PD, and chronic environmental exposure to MPP+ or rotenone is unlikely to cause PD *per se*. Another possibility is that an endogenous toxin may be responsible for PD neurodegeneration. Distortions of normal DA metabolism might create toxic substances (i.e. reactive oxygen species) because of environmental exposures or inherited differences in metabolic pathways (Dauer & Przedborski 2003; Sandy et al. 1996).

Several environmental contaminants, known to cause nigrostriatal damage at toxic concentrations, could contribute to PD by interference with mitochondrial respiratory functions and ROS generation (Di Monte 2003, Jenner 2003). The insecticides PQ, rotenone and the fungicide 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. Exposure to various metals has long been suggested to increase risk of PD, based on experimental studies, but human evidence remains inconclusive (Lai et al. 2002; Tanner et al. 2014).

3.3.1 Heavy metals and PD
Iron

Iron (Fe2+), the most abundant metal present in the human brain, has been proposed to play a role in the pathogenesis of PD for its pro-oxidants characteristics that may lead to ROS generation (Sian-Hulsmann et al., 2011). Several studies have focused on the ability of Fe2+, as well as other metals to facilitate the aggregation of the PD-related protein α−synuclein, but very few studies investigated the impact of early exposure to iron on development of PD hallmarks in adult life. In the study by Fredriksson and coworkers (1999) at the age of 3 months, offspring exposed in utero to Fe2+ (3.7 or 37.0 mg/kg/bw) on GD 10-12, showed marked hypokinesia and lack of habituation in spontaneous activity. These effects were more pronounced in mice treated with the higher dose that showed also a dose-dependent impaired performance in the radial arm maze. Brain Fe2+ content was significantly increased in the basal ganglia, but not in the frontal cortex at the higher dose group. In a second study from the same group (Fredriksson et al. 2000) mice were treated in the neonatal stage with Fe2+ (7.5 mg/kg/bw) on either PND 3-5, 10-12 or 19-21. Adult mice treated with Fe2+ on PND 3-5 and 10–12 showed effects similar to those found after gestational exposure on spontaneous motor behavior and learning performance, more marked in animals treated at PND 10-12.

The role of Fe2+ in oxidative stress and superoxide dismutase (SOD) activity may be

implicated in the adverse delayed effects of this metal (Dal Pizzol et al. 2001). Male rats were treated orally with Fe2+ (7.5 or 15 mg/kg) from PND 10 to 12, measuring at 3 months different indexes of oxidative stress. Notably and according to previous clinical and experimental studies, this study demonstrated that Fe2+ exposure during a critical neonatal period induced oxidative stress and modulated superoxide dismutase (SOD) activity restricted to SN in adult rats. Finally, Kaur et al. (2007) investigated the role of early exposure of Fe2+ at a dose (120 µg/g of carbonyl iron/BW) equivalent to that found in iron-fortified human infant formula as a risk factor for PD-like neurodegeneration. Neonatally exposed mice were challenged at adulthood with the toxin MPTP that induces depletion of neostriatal dopamine. Neonatal Fe2+ feeding alone did not induce decrease in ST dopamine levels in 2-month-old mice, also when they were challenged with MPTP. However, at 12, 16 and 24 months of age mice treated with Fe2+ showed more marked DA depletion following acute administration of MPTP than vehicle treated mice. It is worth noting that Fe2+ is important for many biological reactions such as the synthesis and release of neurotransmitters. In particular, the neonatal period is critical for the establishment of normal Fe2+ content in the adult brain and its regional distribution; deficiency such as excess of this metal can perturb the typical neurological development. These results highlight the need of epidemiological research in humans, especially on the potential long-term effects of Fe2+ supplementation during infancy (i.e. milk) on neurological functions in adulthood.

Manganese

Manganese (Mn) was related to PD since 1837, when it was noted that high Mn exposures caused an extra pyramidal syndrome that resembles the dystonic movements associated with parkinsonian symptoms (Couper 1837; Jankovic 2005). Several evidence on the influence of Mn on neurodegenerative processes has been collected in *in vitro* models. A single study comparing *in vitro* and *in vivo* experiments showed that oxidative stress, mitochondrial dysfunction and neuroinflammation are implicated in Mn-induced neurodegeneration (Milatovic et al. 2009).

Two experimental studies suggest that early Mn exposure might have profound effects on later-life susceptibility to neurotoxins and subsequent degeneration (Cordova et al. 2012, 2013). Cordova et al. (2012) show for the first time *in vivo* the link between early Mn exposure (from PND 8 to 12) and the modulation of intracellular signalling pathways. Oxidative stress, DA cell death and later-life impairment in motor function were also observed. In a subsequent study, the same authors investigated the effects of longer exposure to Mn in developing rats. They demonstrated that exposure to Mn (5, 10 and 20mg/kg) from PND 8 to 27 caused significant deficits in motor

coordination and increased signs of oxidative stress in the ST in rats at 3, 4 or 5 weeks of age (Cordova et al., 2013).

3.3.2 Pesticides and PD

One hit studies

Experimental models assessing the causative link between exposure to pesticides and PD-like neurodegeneration have focused on the effects of environmental toxins on the DA nigrostriatal system. This system develops both pre- and postnatally, with receptor development occurring predominantly in the postnatal period (Giorgi et al. 1987; Voorn et al. 1988). As described above, the chemical structure of the insecticide PQ resembles that of MPP+ , the toxic metabolite that mediates the effects of the parkinsonism-inducing agent MPTP. Similarly, administration of PQ to mice produces several neurotoxic effects, including damage of the DA nigrostriatal system (McComark et al. 2002). Dithiocarbamate fungicides also possess DA activity: maneb (MB) administered acutely exacerbates the behavioral symptoms induced by MPTP in mice (hypokinesia and catalepsy). Similar effects have been described with the OC herbicide dieldrin, an inhibitor of mitochondrial respiration.

Richardson et al. (2006) showed that perinatal exposure to low doses of dieldrin (0.3, 1, or 3 mg/kg every 3 days) during gestation and lactation altered the levels of the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) at 12 weeks of age. The alterations of both the DA markers were exacerbated by MPTP injections at 12 weeks. Wilson et al. (2014) investigated the effects of in utero exposure to endosulfan, an OC insecticide, in male offspring at 3 months of age. Female mice received endosulfan (1mg/kg/bw) every 2 days from 2 weeks before breeding until end of lactation. The authors found a reduced expression of DAT and tyrosine hydroxylase (TH) in the striatum (ST) of treated mice, exacerbated by exposure to additional insults such as MPTP. Endosulfan failed to cause the same effects when administered at the same dose and for the same period to adult mice. These findings indicate that gestation and lactation are critical windows of sensitivity to endosulfan exposure and development of nigrostriatal DA system.

Two studies evaluated the effects of developmental exposure to the herbicide atrazine (ATR) during gestation, lactation and pubertal periods on DA system development (Li et al. 2014; Sun et al. 2014). The *in utero* exposure to ATR (25 or 50 mg/kg) from GD 0 to PND 1 reduced the level of DA and expression of orphan nuclear hormone (Nurr1), VMAT2, DAT and TH genes in ST and SN, respectively, at 6 months of age (Li et al., 2014). In a second study, the authors assessed the effects of a more prolonged exposure to ATR given to pregnant mice from GD 5 to PND 22 at the same doses of the previous study: DA concentrations and mRNA levels of Nurr1 were decreased in the

offspring at 12 months of age. Decreased Nurr1 levels paralleled changes in the mRNA levels of VMAT2, which controls the transport and reuptake of DA (Sun et al. 2014).

Two hit studies

In line with the double hit hypothesis, it has been suggested that developmental exposure to either PQ or MB alone, or in combination, would result in permanent nigrostriatal DA system neurotoxicity, and, secondly, it would render this system more susceptible to a second chemical challenge later in life. In the study of Thiruchelvam et al. (2002), mice were exposed to PQ, MB and PQ+MB from PND 5 to 19 and then re-challenged with the single agents or with the combination at 6.5 months of age. Developmental and adult exposure to PQ+MB combination produced the greatest damage of the nigrostriatal DA system and a significant reduction in motor activity. Furthermore, increased sensitivity to the re-challenge with PQ and MB either alone or in combination was observed in only those animals treated neonatally with the combination. Vice versa, exposure to PQ or MB alone produced minimal changes but, after adult rechallenge, a significant decrease in DA levels and nigral cell count was found, confirming that adult re-exposure might un-mask a condition of silent toxicity. Thus, developmental exposures of mice to PQ+MB combination led not only to a permanent and selective loss of DA neurons in the SN*pc* but also enhanced the impact of these pesticides administered during adulthood relative to developmental only or adult only treatment. Exposure to MB alone during gestation resulted in enhanced response to PQ in adulthood, including notable reductions in levels of DA and its metabolites and loss of nigral DA neurons, despite the structural dissimilarity and the different mode of action of PQ and MB (Cory-Slechta et al. 2005).

Another line of research considered the role of *in utero* exposure to MB, combined with subsequent exposure to PQ, in disrupting the nigrostriatal system (Barlow et al. 2004). Pregnant mice were exposed to MB or PQ on GD 10-17, at doses ten times lower than that used in young adult studies; 2-month-old offspring received either PQ or MB at the doses used for adult mice for 8 days. .One week after the termination of the second treatment, mice were assessed in a motor activity task and then sacrificed to measure brain DA markers. The authors observed only in males exposed prenatally to MB and then in the adulthood to PQ, a 95% decrease of locomotor activity, associated to decreased levels of striatal DA, increased striatal DA turnover and selective reduction in DA neurons of the SN*pc*. The authors concluded that the sequence of exposures is critical, since prenatal exposure to PQ followed by adult challenges with MB failed to produce marked alteration of locomotor activity or any DA neuron loss (Barlow et al. 2004).

3.3.3 Maternal infection and PD

Inflammation and reduced antioxidant defences (i.e. reduced glutathione activity) has been implicated in PD pathogenesis (McGeer et al. 2001). Intrauterine infection might lead to nigral cell loss in the fetal brain and subsequent permanent decrease in DA neurons, secondary to inflammation. In such framework, Zhu et al. (2007) assessed the effects of prenatal LPS exposure on glutathione (GSH) metabolism in rat brain. They observed a disturbance of GSH homeostasis in offspring of dams administered with LPS at GD 10.5, which possibly makes DA neurons more susceptible to the secondary chemical insult. Wang et al. (2009) have observed significant reduction of DA and serotonin (5-HT) levels accompanied by loss of DA and 5-HT neurons in SN and in the dorsal raphe nucleus (DRN) in adult rats offspring of pregnant dams exposed to a single injection of LPS (33 μg/KG) at GD 10.5. Fan and coworkers (2011a) assessed the effects of intracerebral injection of LPS (1 mg/kg) in male rat pups at PND 5. Although on PND 70 rats spontaneously recovered the neurobehavioral dysfunction induced by LPS, the authors observed persistent injury to DA system and strong inflammatory response including increased activation of microglia and inflammatory cytokines in the brain. When challenged with METH (0.5 mg/kg), LPS-exposed rats showed increased METH-induced locomotion and stereotyped behaviors as compared to control rats.

3.3.4 Multiple hits in PD: maternal infection and chemical insult combination

Other their lifespan, human beings are exposed to mixtures of chemicals as well as to several non-chemical risk factors. One of the main question in the assessment of the environmental hypothesis of PD etiology is whether sequential exposures to different kinds of risk factors across the lifetime would result in cumulative neurotoxicity to the nigrostriatal DA system. Various models based on "multiple hits" have been developed, assuming that prenatal inflammation makes the brain more susceptible to subsequent exposure to DA neurotoxins.

The first studies combining maternal infection with exposure to an environmental neurotoxicant at a later life stage were carried out by Ling and co-workers (2004). Rats were exposed to LPS prenatally, and then administered with a sub-toxic dose of the DA neurotoxin rotenone (1.25 mg/kg per day for 14 days) when 16-months old. The combined effects of prenatal LPS and postnatal rotenone exposure produced higher DA cell loss compared with the effects of single exposure to either LPS or rotenone. Prenatal LPS exposure also led to increased levels of oxidized proteins and the formation of α-synuclein and eosin-positive inclusions resembling Lewy bodies. In the second study, the same authors (Ling et al. 2004) injected on PND 99 the neurotoxin

6-hydroxydopamine (6-OHDA) or saline into animals exposed to either LPS or saline prenatally. The results showed that animals exposed to prenatal LPS or postnatal 6-OHDA alone had fewer DA neurons than controls, while the two toxins combined produced a greater loss. They conclude that prenatal exposure to LPS produces permanent cell loss accompanied by an inflammatory state that leads to further DA neuron loss once in the presence of subsequent neurotoxin exposure.

Fan et al. (2011b) found that neonatal intracerebral injection of LPS following administration of rotenone at low dose (1.25 mg/kg/bw) for 14 days from PND 70 resulted in PD-like neurobehavioral deficits later in life. In agreement with this hypothesis, Tien et al. (2013) demonstrated that neonatal LPS exposure enhanced the rotenone-induced accumulation of α-synuclein aggregation and DAT protein expression in the SN. Overall, these studies show that brain inflammation in early life may enhance adult susceptibility to develop neurodegenerative hallmarks triggered by environmental toxins.

To compare the effects of gestational LPS exposure with neonatal systemic LPS exposure on adult DA neuron susceptibility to rotenone neurotoxicity, rats was administered with LPS at PND 5 (Cai et al. 2013), and, at adulthood, were challenged with rotenone at a dose of 1.25 mg/kg per day for 14 days. Briefly, rotenone induced loss of DA neurons and PD-like motor impairment in 3-month-old rats that had experienced neonatal LPS exposure, but not in those without the LPS exposure. These results support the view that, although neonatal systemic LPS exposure may not necessarily lead to loss of DA neurons in the SN, it could cause persistent functional alterations that predispose the nigrostriatal system to be damaged by environmental toxins.

Another recent line of evidence concerns air pollution, which has been associated with adverse neurological and behavioral effects in children and adults. Increases in neuroinflammation, oxidative stress, and glial activation have been identified as putative mechanisms by which air pollution exposures may impair central nervous system function in adults. Particulate matter, one of many components of ambient outdoor air pollution, causes elevation in cytokines and oxidative stress in the brain (Campbell et al. 2009). Given the evidence suggesting susceptibility of both the SN and ST to components of air pollution, two studies assessed whether exposure of mice to concentrated ambient ultrafine particles (CAPS; <100 nm diameter) during the first two weeks of life would alter susceptibility to induction of the PD phenotype by the combined administration of PQ+MB, utilizing 10 mg/kg PQ and 30 mg/kg MB 2× per week for 6 weeks (Thiruchelvam et al. 2000a; Thiruchelvam et al. 2003b). Animals treated with CAPS in the early postnatal period show enhanced response to PQ+MB combination. Both CAPS and PQ+MB elevated glutamate levels in ST, consistent with potential excitotoxicity. These findings demonstrate the ability of postnatal CAPS to produce locomotor dysfunction and dopaminergic and glutamatergic changes, independent

of PQ+MB, in brain regions involved in PD (Allen et al., 2014).

Table 3.a Experimental studies assessing the role of early environmental factors in AD

	Compound	Species	Exposure Window	Outcome in offspring	References
				AD experimental models	
Metals	Pb	Rat	PND 0 to 20	↑APP and Aβ expression	Basha et al. 2005
	Pb	Rat	PND 1 to 20 PND 18 to 20 months	↑oxidative DNA damage NE	Bolin et al. 2006
	Pb	Monkey	PND 0 to 400	↑oxidative DNA damage, ↓DNA methyltransferase, ↑APP, BACE1, and Sp1 mRNA levels	Wu et al. 2008
	Pb	Mouse	GD1 to PND 21	↓learning and memory ability, ↑p-tau and Aβ expression	Li et al. 2010
	Pb	Mouse	GD 13 to PND 20	↓immune response, metal-binding, metabolism and transcription/transduction coupling genes	Dosumnu et al. 2012
	Pb	Monkey	PND 0 to 400	↑tau protein and mRNA levels	Bihaqi & Zawia, 2013
	Pb	Mouse	PND 1 to 20 7 to 9 months	↑tau protein and mRNA levels NE	Bihaqi et al. 2014
	As+Cd+Pb	Rat	GD 5 to PND 180	↑Aβ expression, ↓learning and memory abilities, ↑oxidative stress, ↑inflammatory responses	Ashok et al. 2015
Pesticides	CPF	Tg2576	6 months	↑Aβ expression, ↓spatial retention	Salazar et al. 2011
	CPF	Tg2576	4 to 6 months	NE	Peris-Sampedro et al. 2014
	PQ	Tg2576	3 to 4 months	↓associative learning and memory, ↑Aβ expression, ↑oxidative and mitochondrial damage	Chen et al. 2012
Inflammation	LPS	Mouse	GD 15 to 17	↓learning and memory abilities	Chen et al. 2011
	Poly:C	Mouse	GD 17	↑inflammatory cytokines, ↑APP expression, ↑p-tau expression, ↓working memory	Krstic et al. 2012

NE: No Effects
Tg: transgenic mouse

Tartaglione, Venerosi and Calamandrei, contribution to "Neurotoxin modelling of brain disorders"

Table 3.b Experimental studies assessing the role of early environmental factors in PD

	Compound	Species	Exposure Window	Outcome in offspring	References
				PD experimental models	
Metals	Fe^{2+}	Mouse	GD 10 to 12	↓motor behavior, ↓ spatial learning ability	Fredriksson et al. 1999
	Fe^{2+}	Mouse	PND 3 to 5; PND 10 to 12 PND 19 to 21	↓motor behavior, ↓ spatial learning ability NE	Fredriksson et al. 2000
	Fe^{2+}	Mouse	PND 10 to 17	↑oxidative stress, ↓ DA in ST, ↓DA neurons in SN, ↑neuronal vulnerability to MPTP	Iaur et al. 2007
	Fe^{2+}	Rat	PND 10 to 12	↑oxidative stress, ↓SOD activity	Dal Pizzol et al. 2001
	Mn	Rat	PND 8 to 12	altered intracellular signaling pathways, ↑stress and mitochondrial dysfunction, ↑ caspase activity; ↓ motor alterations	Cordova et al. 2012
	Mn	Rat	PND 8 to 27	altered intracellular signaling pathways, ↑stress and mitochondrial dysfunction, ↑ caspase activity, ↓motor alterations	Cordova et al. 2013
Pesticides	Dieldrin	Mouse	gestation and lactation	↑DAT and VMAT2 levels, ↑neuronal vulnerability to MPTP	Richardson et al. 2006
	Endosulfan	Mouse	2 weeks before breeding until lactation	↓ in DAT and TH in SN, ↑ neuronal vulnerability to MPTP	Wilson et al. 2014
	PQ, MB or PQ+MB	Mouse	6 weeks (1st)	↓ DA and its metabolites in ST, ↓DA neurons in the SNpc	Thiruchelvam et al. 2000
	ATZ	Rat	GD 0 to PND 1	↓DA levels in ST, ↓Nurr1, VMAT2, DAT and TH expression in SN	Li et al. 2014
	ATZ	Rat	GD 5 to PND 22	↓DA levels in ST, ↓Nurr1, VMAT2, DAT and TH expression in SN	Sun et al. 2014
Inflammation	LPS	Rat	GD 10.5	glutathione homeostasis disturbance, ↓DA neurons in the SN, ↑neuronal vulnerability to secondary LPS exposure, ↓neuron in SN and DRN, ↓DA and 5-HT levels	Zhu et al. 2007
	LPS	Rat	GD 10.5	↓DA and 5-HT neurons in SN and DRN, ↓ DA and 5-HT levels	Wang et al. 2009
	LPS	Rat	PND 5	↓motor functions, ↓DA neurons in SN and VTA; ↓microglial activation and inflammatory responses, ↑mitochondrial damage, ↑behavioral reaction to METH	Fan et al. 2011a

NE: No Effects

Table 3.c Experimental studies assessing the role of early environmental factors in PD: multiple hit studies

	Compound	Species	Exposure Window (Multiple Hit)	PD experimental models Outcome in offspring after multiple hit	References
Combined Chemicals	PQ or MB PQ or MB	Mouse	10-17 GD (1st) 6 weeks (2sd)	↓DA neurons in the SNpc, ↓DA level in ST, ↓motor behavior only under combined exposure (MB+PQ)	Barlow et al. 2004
	PQ, MB or PQ+MB PQ, MB or PQ+MB	Mouse	PND 5 to 19 (1st) 6.5–7.5 months (2nd)	↓motor behavior, ↓DA level in ST, ↓DA neurons in the SNpc only under combined exposure	Thiruchelvam et al. 2002
Combined Chemicals and Inflammation	LPS Rotenone	Rat	PND 5 (1st) PND 70 (2nd)	↓motor behavior, ↓DA neurons in the SN, ↑mitochondrial damage, ↑microglial activation, ↑neurobehavioral vulnerability to rotenone exposure	Fan et al. 2011
	LPS Rotenone	Rat	PND 5 (1st) PND 70 (2nd)	↑α-synuclein aggregation, ↑DAT expression, ↑mitochondrial damage	Tien et al. 2013
	LPS Rotenone	Rat	GD 10.5 (1st) PND 21 (2nd)	↓DA neurons in the SN, ↓DA levels in ST, ↑microglial activation, ↑oxidative stress, ↑α-synuclein and eosin positive inclusions	Ling et al. 2004
	LPS Rotenone	Rat	GD 10.5 (1st) PND 99 (2nd)	↓DA neurons in SN and VTA, ↓DA levels in SN, ↑cytokine levels in SN	Ling et al. 2004
	LPS Rotenone	Rat	PND 5 (1st) PND 70 (2nd)	↑brain inflammation, ↓motor behavior, ↑neuronal vulnerability to rotenone exposure	Cai et al. 2013
	CAPS PB+MB	Mouse	PND 4-7 ; PND 10-13 (1st) PND 60 (2nd)	↓DA neurons in ST and SNpc, ↑glutamate levels, ↓motor behavior	Allen et al. 2014

NE: No Effects

4. Concluding remarks

Increasing evidence indicates that events occurring in the earliest stages of human development may influence both resilience and vulnerability to several adult diseases, including neuropsychiatric and neurologic disorders. As stated by Olden (2011) the timing of environmental exposure is a crucial factor that influences the size of the gene-environment interaction: whereas genes may contain the potential for adverse health outcomes, subsequent exposure to environmental triggers is required to initiate physiological or pathological pathways responsible for health and disease. Experimental models confirm this general view. Research in animals has clearly shown that diverse environmental stressors may interfere with typical brain developmental trajectories in critical time windows, inducing silent sub-clinical alterations that may hesitate in pathophysiology and overt functional (e.g. behavioral) deficits at a later life stage. The bulk of data produced by developmental neurotoxicologists since the 1980s has started a series of epidemiological studies to assess the possible link between sub-toxic exposure to chemical pollutants and neurobehavioral effects in children. Thanks to such combined effort, recent advances in research offer important clues into the complex etiology of autism and other neurodevelopmental disorders, indicating that developmental exposure to chemicals cannot be ruled out (Rossignol et al. 2014). Specifically, variations in candidate genes may confer higher individual vulnerability to environmental toxicants. Of note, the convergence of risk factors on the same cell/molecular pathways in critical developmental windows, combined with a vulnerable gene makeup, would trigger the development of the disorders or predispose to adverse health outcome later on. As an example, children at risk for autism may present constitutionally reduced antioxidant defenses: this may render the individual more vulnerable to oxidative stress and inflammatory processes produced by both xenobiotic exposure and maternal infection during pregnancy (Rose et al., 2012).

A similar research effort has not been taking place as concerns the developmental origin of NDDs and the role of environmental factors in increasing risk. As discussed in Chapter 1, the long latency between exposure and disease occurrence, the difficulty of reconstructing exposures that occurred several decades before disease manifestation and the wealth of factors which the individual is exposed to during the life course, makes the developmental origin of NDD a promising but difficulty provable hypothesis. Nonetheless, given the huge public health impact of NDDs, identification of early risk factors would be crucial to design prevention and/or intervention strategies: in this perspective, the comprehension of the environmental etiology of major NDDs requires innovative study paradigms with multifaceted and multidisciplinary approaches.

The experimental findings presented in Chapter 2 prove the utility of *in vivo* models and

support a role of environmental chemicals in increasing risk of NDDs. These experiments showed not only that an environmental insult in early life have long-term, irreversible consequences, but also that the insult must occur during a critical period in development to have its maximal effect. Gestational and neonatal exposure to metals and pesticides as well as experimentally-induced maternal infection both lead to alterations in developmental programming that are expressed as an irreversibly altered function. The functional effects often include significant motor and/or cognitive impairments resembling the pathological phenotypes of major human NDDs. Most of these models present strong construct validity, as the more robust hallmarks of AD and PD are associated with the functional impairment. Furthermore, epigenetic processes seem to play a key role in the mechanisms underlying these phenomena, as shown by the finding that perinatal exposure to Pb modulates the expression of AD-related genes, influencing the course of amyloidogenesis and oxidative DNA damage via DNA methylation (Dosumnu et al. 2012). Among environmental pollutants, Pb is a paradigmatic example of a developmental neurotoxicant able to interfere with developmental programming through different and possibly interrelated mechanisms. These include the antagonism of NMDA receptors in the early developmental phases, which is the key event at the basis of Pb interference with synaptogenesis, neural network formation and behavioral plasticity (Toscano and Guilarte 2005); but Pb also induces latent changes in antioxidant defenses and mitochondrial redox dysfunction that may trigger neurodegeneration-related pathways at later life stages (Caito and Aschner 2015).

The experiments assessing the double or multiple hit hypothesis, either by re-challenging the animal with the same or a different chemical compound at different life stages or by presenting a chemical insult in the adulthood following fetal exposure to maternal inflammation, support the LEARn hypothesis advanced by Lahiri et al (2007). The end-result of the fetal insult in fact is an animal that is sensitized such that it will be more susceptible to diseases later in life, provided the occurrence of a second hit. In such framework, the exposure of the fetal brain to pro-inflammatory cytokines secondary to maternal infection appears as a major causative factor of altered developmental programming and enhanced vulnerability. This supports the view that neurodevelopmental and neurodegenerative disorders do share pathogenic pathways: abnormal immune activation in the early stage of brain development may constitute the common event predisposing to disease at some point in the lifespan of the individual.

The question arising at this point is as to whether experimental findings may help to fill the huge gaps in knowledge that lay beneath the developmental hypothesis for NDDs. Whereas some of the studies here reviewed offer interesting clues for the mechanistic underpinnings of chemical-related effects, experimental models on this topic are still at their beginning. The very nature of the

problem requires the establishment of prospective animal cohorts: this implies the use of developmental exposures with multilevel analysis of the embryos, fetus and pups, as well as of later life stages to trace dysfunction/disease. Research must use environmentally relevant doses, dose response curves and the examination of the relationship between the molecular mechanism proposed and the disease/dysfunction studied. This would also require the use of the new technologies of gene expression profiling and epigenetics: a critical component is the development of a direct correlation and eventually a cause-effect relationship between the alterations in gene expression during development (either increased, decreased or inappropriate timing) to alterations in "omic" signatures that may predict a specific disease or dysfunction later in life. To date no available biomarker is a clear and validated indicator of typical brain development. Recent studies suggest that placental miRNA expression profiles and DNA methylation of specific genes are associated with measures of neurobehavioral outcome in the infants as well as with increased risks of neurological and neurodegenerative diseases (Sheinerman and Umansky 2013); altered Aβ protein in plasma is related with neurodegenerative risk after prenatal Pb exposure (Mazumdar et al. 2012). *In vivo* models could be instrumental to identify robust peripheral biomarkers of exposure, effects and susceptibility, to be subsequently verified in large prospective human cohort studies to establish biologically plausible links between early chemical exposure and later health effects.

Acknowledgments

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