NeuroToxicology 33 (2012) 810–816

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Developmental origins of adult diseases and neurotoxicity: Epidemiological and experimental studies

Donald A. Fox [a,b,c,*], Philippe Grandjean [d,e], Didima de Groot [f], Merle G. Paule [g]

[a] University of Houston, College of Optometry, Houston, TX, USA
[b] University of Houston, Department of Biology and Biochemistry, Houston, TX, USA
[c] University of Houston, Department of Pharmaceutical Sciences, Houston, TX, USA
[d] Department of Environmental Health, Harvard School of Public Health, Boston, USA
[e] Department of Environmental Medicine, University of Southern Denmark, Odense, Denmark
[f] Quality and Safety Research Group, TNO, Zeist, The Netherlands
[g] Division of Neurotoxicology, National Center for Toxicological Research, FDA, Jefferson, AR, USA

ARTICLE INFO

Article history:
Received 28 October 2011
Received in revised form 22 December 2011
Accepted 22 December 2011
Available online 10 January 2012

Keywords:
Methylmercury
Lead
Ketamine
Development
Aging
Behavior
Metabolic syndrome
Obesity
Neurodegeneration

ABSTRACT

To date, only a small number of commercial chemicals have been tested and documented as developmental neurotoxicants. Moreover, an increasing number of epidemiological, clinical and experimental studies suggest an association between toxicant or drug exposure during the perinatal period and the development of metabolic-related diseases and neurotoxicity later in life. The four speakers at this symposium presented their research results on different neurotoxic chemicals relating to the developmental origins of health and adult disease (DOHaD). Philippe Grandjean presented epidemiological data on children exposed to inorganic mercury and methylmercury, and discussed the behavioral outcome measures as they relate to age and stage of brain development. Donald A. Fox presented data that low-dose human equivalent gestational lead exposure produces late-onset obesity only in male mice that is associated with neurodegeneration. Didima de Groot presented results on prenatal exposure of rats to methylazoxymethanol and discussed the results in light of the etiology of western Pacific amyotrophic lateral sclerosis and Parkinson-dementia complex. Merle G. Paule addressed the long-term changes in learning, motivation and short-term memory in aged Rhesus monkeys following acute 24 h exposure to ketamine during early development. Overall, these presentations addressed fundamental issues in the emerging areas of lifetime neurotoxicity testing, differential vulnerable periods of exposure, nonmonotonic dose–response effects and neurotoxic risk assessment. The results indicate that developmental neurotoxicity results in permanent changes, thus emphasizing the need to prevent such toxicity.

1. Introduction

To date, only a small number of commercial chemicals have been tested and documented as developmental neurotoxicants. Moreover, an increasing number of epidemiological, clinical and experimental studies suggest an association between toxicant or drug exposure during the perinatal period and the development of metabolic-related diseases, neurotoxicity and neurodegeneration later in life. Due to the complex and interrelated spatiotemporal development of the central nervous system (CNS), it is likely that developmental insults will result in permanent structural and/or functional alterations during adulthood and/or aging. Compelling epidemiological, pharmacological and toxicological evidence shows that there are several vulnerable periods of growth and development during which environmental interactions with the immune system and genome increase susceptibility to CNS and metabolic diseases during aging (Cameron and Demerath, 2002; Gluckman et al., 2007; Heindell, 2008; Bilbo and Schwarz, 2009; Swanson et al., 2009). These findings define the developmental origins of health and disease (DOHaD) paradigm. Moreover, this concept is extended to included behavioral alterations (Van den Bergh, 2011). The gestational period is particularly sensitive to altered nutritional and hormonal homeostasis, chemical/toxicant exposures and increased proinflammatory cytokines (Rice and Barrone, 2000; Mendola et al., 2002; Taylor and Poston, 2007; Leasure et al., 2008; Newbold et al., 2008; Bilbo and Schwarz, 2009). The four speakers at this symposium presented their research results on different neurotoxic chemicals relating to DOHaD.

* Corresponding author at: University of Houston, College of Optometry, 4901 Calhoun Road, Houston, TX, USA. Tel.: +1 713 713 743 1964.
E-mail addresses: dafox@uh.edu (D.A. Fox), pgrandjean@health.sdu.dk (P. Grandjean), didima.degroot@tno.nl (D. de Groot), merle.paule@fda.hhs.gov (M.G. Paule).

0161-813X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2011.12.016

2. Epidemiological evidence of developmental window of susceptibility to neurotoxicant exposure: age-dependent outcomes (Philippe Grandjean)

Among the small number of environmental chemicals that cause developmental neurotoxicity in humans, methylmercury (MeHg) has an interesting, if not dramatic history, which is still unfolding (Table 1). As MeHg usually originates from seafood, prenatal exposure is generally much higher than early postnatal exposures. The most serious consequences were first seen first in Minamata, Japan, in the 1950s. However, not until 2003 was international consensus achieved (JECFA, 2003), that exposure limits for this neurotoxicant should aim at protecting the developing brain, rather than the nervous system in general, irrespective of age. During recent decades, discussions focused on several sources of uncertainty, which were initially thought capable of erroneously inflating the apparent neurotoxicity findings. These concerns have been clarified and reveal an improved understanding of the developmental neurotoxicity of MeHg.

One issue is the extent and the implications of imprecise exposure assessments in epidemiological studies. Some exposure biomarkers may have a relative imprecision as high as 50%, thereby substantially biasing the dose-effect relationships toward the null (Grandjean et al., 2003). To document exposure-associated neurobehavioral deficits at different ages, we carried out prospective studies in birth cohorts. The first cohort was examined up to three times through 22 years of age. Through this research, several important concerns were addressed including the significance of the age at outcome assessment, the sensitivity of tests to MeHg neurotoxicity and possible differences in vulnerability. The results supported the choice of early school age (i.e., 7 years) as an appropriate stage of development for neurobehavioral assessment. We found that for each doubling in MeHg exposure, the child's brain development was delayed by 1–2 months with some differences between domains. Subsequent studies are in agreement with this initial finding and indicated that the deficits are likely to be permanent (Grandjean and Herz, 2011). This conclusion is also in agreement with a study employing functional MR scanning during simple tasks, where subjects highly exposed to MeHg prenatally revealed abnormalities in the brain areas activated (White et al., 2011). As these changes were present at adolescence, it seems likely that developmental methylmercury neurotoxicity is permanent. However, that does not mean that compensation cannot occur.

To address this question, we compared the difference in performance between ages 7 and 14 years (Debes et al., 2006). The tendencies only approached statistical significance. However, it appeared that those subjects who performed well at age 7 years, no matter their mercury exposure level, improved more than the cohort average during the subsequent 7 years. The opposite was true for subjects with a poor performance at age 7. These findings suggest that the distribution of cognitive skills may not be moved to the left by a neurotoxic exposure, but it may rather be squeezed toward the left, thus preserving most of the children with initial high performance levels.

Another issue of concern was that known or unknown confounders could affect the mercury-associated effects seen in the Faroes and maybe in New Zealand (Crump et al., 1998). Perhaps, co-exposure to PCBs, another marine pollutant, could cause some of the neurotoxicity observed. However, adjustment of the Faroes data for PCB exposure did not eliminate the mercury effects (Grandjean et al., 2003). If these effects were explained by another pollutant (or other confounder, whether chemical, social or genetic), then this parameter should be more closely associated with the cord-blood Hg concentration, as the best risk indicator, than with the maternal hair-Hg to cause the greater effect estimates for the cord-blood level than for the hair level. The confounder also should be a better risk indicator when mothers with variable mercury exposure were excluded. It is difficult to imagine a confounder that would satisfy these requirements (Grandjean and Herz, 2011).

An additional concern involved so-called negative confounding, as MeHg exposure usually originates from fish and seafood that also contains essential nutrients (Choi et al., 2008). Thus, the competing effects of mercury and nutrients needed to be ascertained, so that properly adjusted measures can be generated for each components' effects on the relevant outcomes and neither of them is underestimated. In the Faroes, adjustment for maternal fish intake during pregnancy only resulted in small increases in the calculated MeHg toxicity (Budtz-Jorgensen et al., 2007). The limited effect was due to the poor correlation between fish intake and mercury exposure biomarkers. Information has now become available from the Seychelles (Stokes-Riner et al., 2011), where mutual adjustment for fish intake and MeHg exposure revealed effects of both – in opposite directions – while neither had clear effects without the adjustment. In this case, one could conclude that the mercury exposure deprived the child of the benefits from the seafood nutrients.

Overall, these issues were crucial to the proper appreciation of the dose–response relationships and calculation of exposure limits. Taking into account imprecision and negative confounding would likely cut the lowest current exposure limit – the one used by the U.S. EPA – by 50% or more (Budtz-Jorgensen et al., 2004). In addition to underestimating the developmental neurotoxicity, the increased risk incurred during fetal development was recognized with a delay. Although evidence was first published in 1952 that MeHg was a developmental neurotoxicant, international consensus and regulation only was reached 50 years later (Table 1). Thus, the evolution of insights into MeHg neurotoxicity demonstrates the challenges in documenting neurodevelopmental deficits due to prenatal neurotoxicant exposure (Grandjean and Landrigan, 2006).

Table 1
Time course of insights into methylmercury toxicity and related interventions.

Year	Event
1866	First published record of fatal occupational methylmercury poisoning
1940–1954	Poisoning cases in workers at fungicide production plants
1952	First report on developmental neurotoxicity in two infants
1956	Discovery of a disease of unknown origin in Minamata, Japan
1955–1972	Poisoning epidemics from use of methylmercury-treated seed grain for cooking in Iraq, Guatemala, Pakistan, Sweden, and the USA
1973	Dose–response relationship described in poisoned adults in Iraq
1978	Exposure limit of 3.3 μg/kg per week based on toxicity in adults; Cree children in Canada assessed at low methylmercury exposure
1986	First report on adverse effects in children related to maternal fish intake during pregnancy (New Zealand)
1997	Population study shows adverse effects in children from methylmercury in maternal seafood intake (Faroe Islands)
1998	White House expert workshop identifies uncertainties in evidence
2000	National Research Council (U.S.) supports exposure limit of 0.1 μg/kg per day during development
2003	International exposure limit of 1.6 μg/kg per week to protect the fetus
2004	European expert committee recommends that exposures be "minimized"
2009	International agreement on controlling mercury pollution

Revised from Grandjean et al. (2010).

3. Low-level human equivalent gestational lead exposure is a risk factor for late-onset metabolic syndrome and neurodegeneration in humans and animals (Donald A. Fox)

The incidence of obesity in developed nations has reached epidemic proportions but common sense causes such as increased consumption of calorically-laden foods and decreased physical activity do not completely account for it (Baillie-Hamilton, 2002; Heindell, 2007). The DOHaD Hypothesis states that fetal and neonatal reprogramming of metabolism significantly contributes to an increased risk of cardiovascular and neurodegenerative diseases as well as obesity and related metabolic disorders in adulthood (Barker et al., 2002; Gluckman and Hanson, 2004; Langley-Evans, 2006; Taylor and Poston, 2007; Weiss et al., 2002). Several synthetic organic and inorganic environmental toxicants appear capable of such reprogramming (Baillie-Hamilton, 2002; Heindell, 2007; Landrigan et al., 2005; Newland and Rasmussen, 2000). Heavy metals represent one such class of toxicant since fetal exposure to tin compounds or lead and cadmium produce significantly increased offspring weight on the day of birth, (Antonio et al., 1999; Grün and Blumberg, 2006). Epidemiological studies reveal persistent increases in body mass index (BMI) during childhood and subsequently young adulthood following moderate-level gestational lead exposure (GLE) and postnatal lead exposure. Similarly, the adverse effects of developmental lead exposure on cognitive, auditory and retinal function, visual-motor function, and motor function and coordination are well-documented in children and developing experimental animals (Altmann et al., 1998; Canfield et al., 2003; Fox et al., 1997, 2008, 2011; Giddabasappa et al., 2011; He et al., 2003; Lilienthal et al., 1988, 1994; Osman et al., 1999; Rothenberg et al., 2000, 2002; Nagpal and Brodie, 2009; Wasserman et al., 2000). However, the long-term effects of GLE or early postnatal lead exposure on these measures are mostly unexplored (Leasure et al., 2008).

The experiments described herein were designed to investigate the long-term effects of low- (peak blood lead concentrations ([BPb]) ≤ 10 μg/dL), moderate- (peak [BPb] > 10 and ≤ 25 μg/dL) and high-level (peak [BPb] > 26 μg/dL) human equivalent gestational lead exposure on year-old C57BL/6 male and female mice. Gender differences were examined because early developmental lead exposure produces an increased risk for attention, visual motor and fine-motor deficits in males (Baghurst et al., 1992; Bhattacharya et al., 1990, 2006; Ris et al., 2004) and male and female animals exhibit differences in metal disposition and lead neurotoxicity (Cory-Slechta et al., 2004; Vahter et al., 2007). C57BL/6 female mice were exposed to low (27 ppm), moderate (55 ppm) or high (109 ppm) lead containing drinking water throughout gestation and until postnatal day 10 (PN10). [BPb] in control, low-, moderate- and high-dose GLE mice was ≤ 1, ≤ 10, ~ 25 and ~ 40 μg/dL, respectively, on PN10 and by PN30 and at 12 months of age all were ≤ 1 μg/dL as described (Giddabasappa et al., 2011; Leasure et al., 2008). Water, food, and dams' weight as well as offspring measures were monitored throughout pretreatment, mating and pregnancy, after delivery and until tissue collection at PN60 (adult). No significant treatment-related differences were observed (Leasure et al., 2008). Four major outcome measures were assessed: body weight, cumulative Wahman running wheel activity for five consecutive nights, cumulative spontaneous activity during 30 min in an Optovarimax behavioral monitor after 15 min of acclimation, motor coordination assessed on a Columbus rotarod after training as described (Leasure et al., 2008).

GLE had no significant effect on male or female body weight at PN0, PN10, PN60 or PN180, although by PN60 males in all groups weighed significantly more than females (Leasure et al., 2008). In contrast, Fig. 1A shows that year-old low-dose (+26%), moderate-dose (+21%) and high-dose (+13%) male GLE mice weighed significantly more than age-matched controls (Leasure et al., 2008). Moreover, GLE produced nonmonotonic dose-dependent responses since the alterations were consistently larger in the low-dose, than high-dose, GLE group. These responses are characteristic of inverted U-shaped dose–response curves often observed in lead neurotoxicity studies (Davis and Svendsgaard, 1990). Interestingly, as described (Leasure et al., 2008), there were no significant treatment-related effects of GLE on the body weight of 12 month-old female mice (Fig. 1A). Thus, GLE produced late-onset obesity in male, but not female, mice. The gender selective effect is consistent with reports that boys have increased susceptibility to neurobehavioral alterations and cognitive deficits produced by low- to moderate-level GLE compared to age-matched girls (Bhattacharya et al., 1990, 2006; Ris et al., 2004).

Since 12 month-old GLE males weighed more than age-matched control males (Fig. 1A), the possibility that excess body

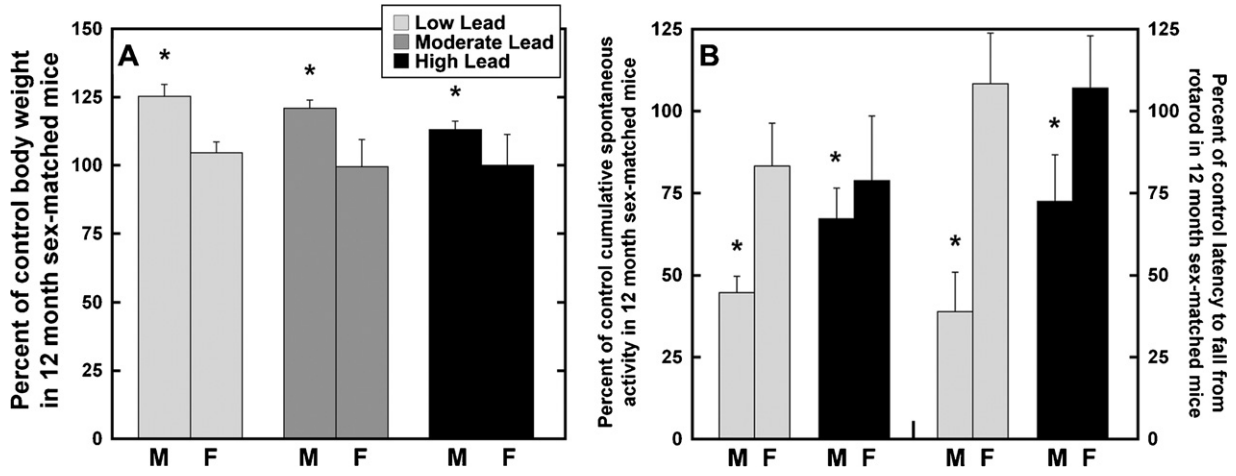

Fig. 1. Percent control body weight, cumulative spontaneous activity and motor coordination in 12 month-old male and female mice following human equivalent gestational lead exposure. (A) Twelve month-old male mice in the low, moderate and high lead-dose groups weighed significantly more than age-matched controls: 26%, 21% and 13%, respectively. Gestational lead exposure produced nonmonotonic dose-dependent responses. There were no significant treatment-related differences in body weight in female mice. (B) Twelve month-old male mice in the low and high lead-dose groups exhibited significantly less cumulative spontaneous activity than age-matched controls. The mean latency to fall from the rotarod significantly decreased in 12 month-old males in the low and high lead-dose groups compared to age-matched controls. Gestational lead exposure produced inverted dose-dependent responses. There were no significant treatment-related differences in cumulative spontaneous activity or mean latency to fall from the rotarod in female mice. $^{*}p < 0.05$ compared to sex-matched controls. Figure adapted from Leasure et al. (2008).

weight made the GLE males less active in their home cages was investigated. This hypothesis was not confirmed as there were no treatment-related differences in male running wheel activity in their home cages over a period of five consecutive dark cycles (Leasure et al., 2008). To determine if there were treatment-related differences in spontaneous (or exploratory) activity, 12 month-old males from control, low-dose and high-dose GLE male mice were examined for 30 min, following a 15 min acclimation period, in an Optovarimax behavioral monitor (Leasure et al., 2008). The 12 month-old male mice in the low-dose and high-dose GLE groups exhibited significantly less cumulative spontaneous activity than age-matched controls: −52% and −35%, respectively (Fig. 1B: left panel). The low-dose GLE males were significantly less active than high-dose GLE males. In contrast, there were no treatment-related differences for female mice (Fig. 1B: left panel).

To investigate the long-term effects of GLE on inter-limb balance and motor coordination, rotarod behavior was assessed. The mean latency to fall from the rotarod was decreased significantly in 12 month-old GLE males compared to age-matched controls (Fig. 1B: right panel). In contrast, the mean latency to fall from the rotarod was not significantly different in 12 month-old female control and GLE mice (Fig. 1B: right panel). The results in 12 month-old male GLE mice are consistent with "negative" dose–response curves since high-dose GLE produced less deviation from control than did low-dose GLE (Davis and Svendsgaard, 1990). Consistent with the negative dose–response curve, rotarod activity was unchanged in adult male rats exposed to 5- to 40-fold higher lead levels during gestation and lactation or lifetime (Ma et al., 1999; Moreira et al., 2001).

The most compelling finding is that GLE is a delayed obesogen and that the weight gain was greater in low- and moderate-dose, than high-dose, GLE mice. These nonmonotonic obesogenic effects appear selective for GLE since low- or moderate-level postnatal lead exposure with similar [BPb] did not alter body weights in developing or 12 month-old mice (Leasure et al., 2008), lifetime exposure to 5- to 20-fold higher lead levels did not affect body weight of 14 month-old male or female rats (Verlangieri, 1979).

The rotarod results are reminiscent of the poorer fine motor control, visual motor function and postural balance found in children, adolescents and young adults with low-to-moderate developmental lead exposure (Baghurst et al., 1992; Bhattacharya et al., 1990, 2006; Ris et al., 2004; Wasserman et al., 2000). Interestingly, there is an increased risk of neuromotor deficits in males (Ris et al., 2004). Our results suggest that low-level GLE contributes to persistent neuromotor and balance deficits, and may be a risk factor for injuries in older males.

In summary, these results demonstrate that GLE mice with peak [BPb] $\leq$ 10 μg/dL, the current low-level of concern (CDC, 1991), have permanent sex-specific motor abnormalities and late-onset obesity. The nonmonotonic dose-dependent responses reveal that low-level GLE produces the most adverse effects. These data raise complex issues for risk assessment and indicate that lifetime measures of dose–response toxicant exposure should be a component of the neurotoxic risk assessment process.

4. Maternal chemical exposure forms the basis of persistent neural impairment in young and adult offspring: a rat model with methylazoxymethanol (MAM) (Didima de Groot)

Animal studies have shown that early exposure to environmental chemicals may reprogram (neuro)physiological set-points and thereby increase susceptibility to diseases later in life. An example is the western Pacific amyotrophic lateral sclerosis and Parkinson-dementia complex (ALS–PDC) that appeared in the genetically distinct populations of Guam, Kii and West Papua. Kisby and Spencer (2011) postulated that a common, yet disappearing, environmental factor played a pivotal role in the etiology of ALS–PDC in these three regions. All used the cycad seed kernel that contains neurotoxic and genotoxic chemicals such as cycasin and MAM for food and/or medical treatment. MAM is a potent neurotoxin taken up by the brain and metabolized to compounds that act as DNA alkylating agents (Johnston et al., 1979; Kisby et al., 2011). Numerous studies have shown that gestational MAM exposure (single high dose of 20–25 mg/kg on one gestational day (GD) between GD14 and GD17) produces delays in reflex development; dysgenesis in different brain regions undergoing proliferation; and cognitive, motor and schizophrenic-like behavioral deficits in rat offspring depending upon the gestational age of exposure (Balduini et al., 1986, 1991; Colacitti et al., 1999; Hradetzky et al., 2012; Johnston et al., 1979; Kisby et al., 2011). However, the long-term morphological and functional effects on offspring following lower doses of MAM, with few exceptions (De Groot et al., 2005a,b,c, 2006), have not been investigated.

Based on a literature review and their experiments with 11 week-old male mice given a single 20 mg/kg dose of MAM, Spencer and colleagues (Kisby and Spencer, 2011; Kisby et al., 2011) suggest that early exposure to MAM disrupts cell signaling and produces long-latency neurodegenerative effects on non-dividing CNS neurons. This hypothesis prompted us to re-evaluate our dose–response neuropathology studies on weanling (postnatal day 22: PN22) and adult (PN62) rats following low dose MAM on GD13–15 (De Groot et al., 2005a,b,c, 2006).

Female rats were injected (ip) on GD13–15 with 0, 1.25, 2.5, 5.0 or 7.5 mg/kg MAM. Two subsets of F1-offspring were selected for neuropathology on PN22 (subset 1) and PN62 (subset 2). Brains were dissected along specific neuroanatomical landmarks and eight brain regions were examined. On PN22 and PN62, neuropathology analyses were carried out at the macroscopic level (gross abnormalities, absolute and relative brain weight) and microscopic level (slide reading and linear morphometry: measurement of brain region/layer width). On PN22, the number of hippocampal pyramidal neurons and cerebellar granular neurons were estimated by stereological methods. On PN22, a marked size reduction of the cerebrum at the site of the pineal body was observed in animals in the 7.5 mg/kg MAM group and in some 5 mg/kg MAM rats. These decreases were still present at PN62. Gross brain abnormalities were limited to this microencephaly and no tumors were found. No gross abnormalities were seen in other organs. Significant dose-related reductions in the absolute and relative brain weights were observed on PN22 and PN62. The anterior and posterior cerebrum were affected, but not the cerebellum: consistent with the postnatal development of the granule cells of the cerebellum (Rodier, 1980).

Examination of hematoxylin and eosin (H&E) stained sections revealed that there were no changes in layer composition or ectopic cells in any of the eight brain regions on PN22 or PN62. Morphometric analysis revealed changes in the cerebrum, frontal cortex, parietal cortex and hippocampus that were consistent with a macroscopic reduction in size. These areas are in the proliferation phase during the gestational time MAM was given to the dam (Rodier, 1980): consistent with MAM acting as a mitotic inhibitor (Johnston et al., 1979). In contrast, the cerebellar layers were not affected. These morphometric results were supported by the stereology results showing a significantly decreased number of hippocampal neurons in the 7.5 mg/kg MAM group, whereas the number of cerebellar granular neurons was unaffected. Spontaneous motor activity showed hypoactivity on PN13, hyperactivity on PN17 and PN21, and no significant differences on PN61 (De Groot et al., 2005a,b,c, 2006).

Kisby and Spencer (2011) postulated that the molecular pathogenesis of toxicant-induced brain diseases can be "self-limiting" or "self-propelling". They state that self-limiting diseases

likely reach a plateau and persist (in the case of cell loss) or possibly reverse. These result from the acute interference with one of more neurobiological processes such as binding to membrane receptors or channels, interference with mitochondrial function, disturbance of synaptic integrity and/or disruption of axonal transport. The resulting cell impairment produces functional changes that may ultimately become manifest as clinical or subclinical symptoms that produce "self-limiting" neurological diseases. In contrast, "self-propelling" or progressive neurological diseases do not become manifest when such "classical" neurobiological processes form the basis of toxicant-induced diseases. Rather, they worsen as the cellular and molecular substrates are different. They argue, based on a high single dose of MAM to adult mice and gene expression data (Kisby et al., 2011), that MAM produces a "propelling" (progressive) neurological disease since gene network analysis suggested that more than just classical molecular pathogenesis occurred. We will discuss our results in the context of this hypothesis.

The results of our rat study on low-dose prenatal MAM exposure showed significant structural changes (i.e., cell loss) in the brain: especially in the parietal cortex and hippocampus that were proliferating during the MAM exposure (Rodier, 1980). The effects persisted and did not reverse because similar effects were observed at PN22 and PN62. This is consistent with the cortical atrophy produced by MAM on GD15 (Johnston et al., 1979) and shows that prenatal exposure to MAM induced a non-progressive cell-reduction in proliferating regions such as the hippocampus and parietal cortex, but not in the non-proliferating cerebellum. The motor activity testing suggests that MAM produced a developmental delay rather than a permanent locomotor impairment (De Groot et al., 2005c, 2006), as the rodent parietal cortex and hippocampus are involved in spatial exploratory behavior (Torrealba and Valdes, 2008). In classical pathogenesis theory, the consequence of cell reduction is that the neuronal dysfunction persists. In our study, the structural impairment on PN62 resembled that on PN22. Moreover, the lack of motor impairment on PN61, compared to PN17 and PN21 suggests either a developmental delay or neuronal plasticity. Thus, we conclude, that under our experimental conditions, progressive or "self-propelling" genotoxic and neurotoxic effects of MAM did not occur. Our findings can be explained by a temporary arrest of cell proliferation during the gestational MAM exposure window with no further effects afterwards. Whether a different vulnerability window and/or a higher dose of MAM would induce genotoxicity and chronic neurotoxicity are fruitful areas for future research. Finally, and relevant to DOHaD and the theme of the symposium, it is evident from these experiments that prenatal exposure to low and environmentally relevant doses of neurotoxic chemicals in the food can have long-lasting effects on health and disease in later life.

5. Acute neonatal exposure to ketamine and long-lasting deficits in learning, motivation and concept formation in rhesus monkeys (Merle G. Paule)

Our increased ability to keep premature and compromised infants alive results in an ever-increasing population in our nation's neonatal intensive care units. Part of this success lies in the increased number of complicated surgical and other interventions that occur in this already-at-risk population. Many of these are conducted under various forms of anesthesia and/or sedation. Concerns over the potential adverse effects of these kinds of drug exposures prompted the need for experimental studies to address this important issue.

Blockade of *N*-methyl-D-aspartate (NMDA) receptors by the anesthetic ketamine causes robust increases in apoptotic cell death during the rat brain growth spurt (Hayashi et al., 2002; Ikonomidou et al., 1999; Scallet et al., 2004; Shi et al., 2010; Zou et al., 2009a,b). Excitatory amino acids play critical roles during development by regulating neuronal survival, axonal and dendritic structure, synaptogenesis and plasticity (Komuro and Rakic, 1993; McDonald and Johnston, 1990; Reiprich et al., 2005). Excitatory amino acid receptors also play important roles in long-term potentiation (Collingridge and Lester, 1989), which is important for learning and memory processes (D'Souza et al., 1992; Huang and Stevens, 1998; Tomita et al., 1990). The infant brain is thought to be more sensitive to NMDA receptor manipulation than the adult brain (Bittigau et al., 1999; D'Souza et al., 1992).

Studies in rats exposed to a single episode of an anesthesia using a cocktail typical of those used in children demonstrated subsequent deficits in learning behaviors when tested as young adults (Jevtovic-Todorovic et al., 2003). Thus, it was important to determine if similar phenomena also occurred in nonhuman primates. Ketamine-induced increases in abnormal cell death were shown to occur in the rhesus monkey (Slikker et al., 2007) from the middle of the third trimester of pregnancy to postnatal days 5/6 (not seen by PND 35) (Brambrink et al., 2010; Zou et al., 2009a,b). Next, we determined if there were associated functional consequences in primates, as demonstrated in rodents, with a goal toward making predictions about the effects of ketamine-induced general anesthesia during development on later cognitive function in humans. Several cognitive function tasks from the National Center for Toxicological Research (NCTR) Operant Test Battery (OTB) (Paule et al., 2011) were utilized. These included assessments of motivation, color discrimination, learning, and short-term memory as described (Paule et al., 2011).

Task metrics included percent task completed, response rate and accuracy. Earlier studies (Paule et al., 1999) demonstrated that, in children, several metrics of these OTB tasks correlate positively with IQ scores, thus, their relevance. Earlier studies also demonstrated that, in general, the OTB performance of well-trained monkeys is not different from that of children four to thirteen years of age, with the degree of similarity depending upon task and endpoint (Paule et al., 1990). The translatability of monkey OTB data to humans is further supported by drug studies demonstrating that, where comparable data exist, psychotropic drug effects are the same or similar in both monkeys and humans (Paule, 2001).

Six neonatal rhesus monkeys (PND 5 or 6) were exposed to 24 h of iv ketamine-induced anesthesia. Six controls were unexposed, but separated from their mothers for the same amount of time as the treated animals. Animals reared by their natural mothers without incident were weaned at six months of age. At seven months of age, all animals began training in the NCTR OTB. For subjects to come to understand the rules governing particular OTB tasks, extensive training is required. During this training scores are assigned as animals demonstrate mastery of specifics. The OTB training scores of the ketamine-exposed animals closely tracked those of the controls until short-term memory task training began. This occurred about three months into training when the animals were approximately 10 months of age. This stage of training involves developing the concept of 'matching', whereby subjects are reinforced for 'matching' a previously shown 'sample' stimulus (Paule et al., 2011). By the time ketamine-exposed animals mastered the memory task concept, they were three months behind the control animals. During mastery of learning task performance, the performance of controls and exposed animals was about the same for the first 10 months of training (~17 months of age), after which the performance of controls was significantly better than that of exposed animals. This disparity continued throughout the remainder of the observation period: the following two years (Paule et al., 2011). The deficits manifest

in the form of reduced response rate, accuracy and percent task completed. Similar persistent deficits were observed for response rates in both motivation and color and position discrimination tasks (Paule et al., 2011).

These results provide proof of concept that a single episode of ketamine-induced general anesthesia during a sensitive period of brain growth can cause subsequent cognitive deficits in nonhuman primates. These effects are very long-term and possibly permanent. This observation is particularly troubling when one considers that they are seen in behaviors thought to reflect aspects of brain function related to intelligence in children. The findings from this study represent an example of chemically-induced mental retardation whereby the insult occurred during development and the 'disease' remained evident into adulthood.

6. Conclusions

The four presentations illustrate key aspects of developmental neurotoxicity. As clearly demonstrated by the experience with methylmercury, lead and MAM, structural, functional and/or behavioral deficits induced by exposure to these environmental or dietary toxicants during early development are persistent and impact the long-term health and quality of life. Animal models exist to assess developmental neurotoxicity, but only a small number of chemicals have been examined for this potential. The risks are not limited to environmental chemicals, but also extend to drugs and potential neurotoxic chemicals in food. The results presented illustrate the need to conduct lifetime neurotoxicity and behavioral testing following low-level exposures that occur during highly sensitive periods of early brain development. These outcome measures should be a component of the neurotoxic risk assessment process. Furthermore, understanding the cellular and molecular mechanisms of these low level toxicant exposure will provide fundamental and new information about the normal development of the central nervous system.

Conflicts of interest statement

None of the authors has competing financial interests.

Acknowledgments

Supported in part by Grants RO1 ES09797 (PG), RO1ES012482 (DAF), Core Grant EY07551 (DAF), America Chemistry Council Project ID: NTX0001 (DdG); Dutch Ministries of Health, Welfare and Sports (DdG), Dutch Ministry of Social Affairs (DdG), NICHD (MGP), CDER/FDA (MGP), and NCTR/FDA (MGP). The authors also acknowledge the financial support of the local organizers. The findings and conclusions in the report by MG Paule are those of the author and do not necessarily represent the views of the FDA.

References

Altmann L, Sveinsson K, Krämer U, Weishoff-Hoube'n M, Turfeld M, Winneke G, et al. Visual functions in 6-year-old children in relation to lead and mercury levels. Neurotoxicol Teratol 1998;20:9–17.

Antonio MT, Corpos I, Leret ML. Neurochemical changes in newborn's brain after gestational cadmium and lead exposure. Toxicol Lett 1999;104:1–9.

Baghurst PA, McMichael AJ, Wigg NR, Vimpani GV, Robertson EF, Roberts RJ, et al. Environmental exposure to lead and children's intelligence at the age of seven years. The Port Pirie Cohort Study. N Engl J Med 1992;327:1279–84.

Baillie-Hamilton PF. Chemical toxins: a hypothesis to explain the global obesity epidemic. J Altern Complement Med 2002;8:185–92.

Balduini W, Cimino M, Lombardelli G, Abbracchio MP, Peruzzi G, Cecchini T, et al. Microencephalic rats as a model for cognitive disorders. Clin Neuropharmacol 1986;9(Suppl. 3):S8–18.

Balduini W, Lombardelli G, Peruzzi G, Cattabeni F. Treatment with methylazoxymethanol at different gestational days: physical, reflex development and spontaneous activity in the offspring. Neurotoxicology 1991;12:179–88.

Barker DJ, Eriksson JG, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol 2002;31:1235–9.

Bhattacharya A, Shukla R, Bornschein RL, Dietrich KN, Keith R. Lead effects on postural balance of children. Environ Health Perspect 1990;89:35–42.

Bhattacharya A, Shukla R, Dietrich KN, Bornschein RL. Effect of early lead exposure on the maturation of children's postural balance: a longitudinal study. Neurotoxicol Teratol 2006;28:376–85.

Bilbo SD, Schwarz JM. Early-life programming of later-life brain and behavior: a critical role for the immune system. Front Behav Neurosci 2009;3:14.

Bittigau P, Sifringer M, Pohl D, Stadthaus D, Ishimaru M, Shimizu H, et al. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. Ann Neurol 1999;5:724–35.

Brambrink AM, Back SA, Avidan MS, Creeley CE, Olney JW. Ketamine and isoflurane anesthesia triggers neuronal and glial apoptosis in the neonatal Macaque. Proc Am Soc Anesth 2010 Abs #A375.

Budtz-Jorgensen E, Keiding N, Grandjean P. Effects of exposure imprecision on estimation of the benchmark dose. Risk Anal 2004;24:1689–96.

Budtz-Jorgensen E, Grandjean P, Weihe P. Separation of risks and benefits of seafood intake. Environ Health Perspect 2007;115:323–7.

Cameron N, Demerath EW. Critical periods in human growth and their relationship to diseases of aging. Am J Phys Anthropol Suppl 2002;35:159–84.

Canfield RL, Henderson CR Jr, Cory-Slechta DA, Cox C, Jusko TA, Lanphear BP. Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. N Engl J Med 2003;348:1517–26.

CDC. Preventing lead poisoning in young children: a statement by the Centers for Disease Control and Prevention. US Department of Health and Human Services, Public Health Service; 1991.

Colacitti C, Sancini G, DeBiasi S, Franceschetti S, Caputi A, Frassoni C, et al. Prenatal methylazoxymethanol treatment in rats produces brain abnormalities with morphological similarities to human developmental brain dysgeneses. J Neuropathol Exp Neurol 1999;58:92–106.

Collingridge GL, Lester RA. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev 1989;41:143–210.

Cory-Slechta DA, Virgolini MB, Thiruchelvam M, Weston DD, Bauter MR. Maternal stress modulates the effects of developmental lead exposure. Environ Health Perspect 2004;112:717–30.

Choi AL, Cordier S, Weihe P, Grandjean P. Negative confounding in the evaluation of toxicity: the case of methylmercury in fish and seafood. Crit Rev Toxicol 2008;38:877–93.

Crump KS, Kjellström T, Shipp AM, Silvers A, Stewart A. Influence of prenatal mercury exposure upon scholastic and psychological test performance: benchmark analysis of a New Zealand cohort. Risk Anal 1998;18:701–13.

Davis JM, Svendsgaard DJ. U-shaped dose–response curves: their occurrence and implications for risk assessment. J Toxicol Environ Health 1990;30:71–83.

Debes F, Budtz-Jørgensen E, Weihe P, White RF, Grandjean P. Impact of prenatal methylmercury toxicity on neurobehavioral function at age 14 years. Neurotoxicol Teratol 2006;28:363–75.

De Groot DMG, Bos-Kuijpers MHM, Kaufmann WSH, Lammers JHCM, O'Callaghan JP, Pakkenberg B, et al. Regulatory developmental neurotoxicity testing: a model study focusing on conventional neuropathology endpoints and other perspectives. Environ Toxicol Pharmacol 2005a;19:745–55.

De Groot DMG, Hartgring S, van de Horst L, Moerkens M, Otto M, Bos-Kuijpers MHM, et al. Two- and 3-D assessment of neuropathology in rat brain after prenatal exposure to methylazoxymethanol: a model for developmental neurotoxicty. Reprod Toxicol 2005b;20:417–32.

De Groot D, Hartgring S, van Dael M, Otto M, van der Horst L, Pelgrim M, et al. Motor activity test for developmental neurotoxicity: a usefull test? Reprod Toxicol 2005c;20:464.

De Groot D, Otto M, van der Horst L, Pelgrim M, Lammers J, Bos-Kuijpers M, et al. Developmental neurotoxicity testing: motor activity results considered from body weight and neuropathology perspective. Reprod Toxicol 2006;22:268.

D'Souza SW, McConnell SE, Slater P, Barson AJ. *N*-methyl-D-aspartate binding sites in neonatal and adult brain. Lancet 1992;339:1240.

Fox DA, Campbell ML, Blocker YS. Functional alterations and apoptotic cell death in the retina following developmental or adult lead exposure. Neurotoxicology 1997;18:645–64.

Fox DA, Hamilton RW, Johnson JE, Xiao W, Chaney S, Mukherjee S, et al. Gestational lead exposure selectively decreases retinal dopamine amacrine cells and dopamine content in adult mice. Toxicol Appl Pharm 2011;256:258–67.

Fox DA, Kala SV, Hamilton WR, Johnson JE, O'Callaghan JP. Low-level human equivalent gestational lead exposure produces supernormal scotopic electroretinograms, increased retinal neurogenesis and decreased dopamine utilization in rats. Environ Health Perspect 2008;116:618–25.

Giddabasappa A, Hamilton WR, Chaney S, Xiao W, Johnson J, Mukherjee Fox DA. Low-level gestational lead exposure increases retinal progenitor cell proliferation and rod photoreceptor and bipolar cell neurogenesis in mice. Environ Health Perspect 2011;119:71–7.

Gluckman PD, Hanson MA, Beedle AS. Early life events and their consequences for later disease: a life history and evolutionary perspective. Am J Hum Biol 2007;19:1–19.

Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res 2004;56:311–7.

Grandjean P, Budtz-Jorgensen E, Keiding N, Weihe P. Underestimation of risk due to misclassification. Eur J Oncol (Suppl. 2):2003;165–72.

Grandjean P, Landrigan PJ. Developmental neurotoxicity of industrial chemicals. Lancet 2006;368:2167–78.

Grandjean P, Satoh H, Murata K, Eto K. Adverse effects of methylmercury: environmental health research implications. Environ Health Perspect 2010;118:1137–45.
Grandjean P, Herz K. Methylmercury and brain development: imprecision and underestimation of developmental neurotoxicity in humans. Mt Sinai J Med 2011;78: 107–18.
Grün F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 2006;147:S50–5.
Hayashi H, Dikkes P, Soriano S. Repeated administration of ketamine may lead to neuronal degeneration in the developing rat brain. Paediatr Anaesth 2002;12: 770–4.
He L, Perkins GA, Poblenz AT, Harris JB, Hung M, Ellisman MH, et al. Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. Proc Natl Acad Sci USA 2003;100:1022–7.
Heindell JJ. Animal models for probing the developmental basis of disease and dysfunction paradigm. Basic Clin Pharmacol Toxicol 2008;102:76–81.
Heindell JJ. Role of exposure to environmental chemicals in the developmental basis of disease and dysfunction. Reprod Toxicol 2007;23:257–9.
Hradetzky E, Sanderson TM, Tsang TM, Sherwood JL, Fitzjohn SM, Lakics V, et al. The methylazoxymethanol acetate (MAM-E17) rat model: molecular and functional effects in the hippocampus. Neuropsychopharmacology 2012;37:364–77.
Huang EP, Stevens CF. The matter of mind: molecular control of memory. Essays Biochem 1998;3:165–78.
Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999;83:70–4.
JECFA. Summary and conclusions. Sixty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives, Rome, June 2003.
Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003;23:876–82.
Johnston MV, Grzanna R, Coyle JT. Methylazoxymethanol treatment of fetal rats results in abnormally dense noradrenergic innervation of neocortex. Science 1979; 203:369–71.
Kisby GE, Spencer PS. Is neurodegenerative disease a long-latency response to early-life genotoxin exposure. Int J Environ Res Public Health 2011;10:3889–921.
Kisby GE, Fry RC, Lasarev MR, Bammler TK, Beyer RP, Churchwell M, et al. The cycad genotoxin MAM modulates brain cellular pathways involved in neurodegenerative disease and cancer in a DNA damage-linked manner. PLoS One 2011;6:e20911.
Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 1993;260:95–7.
Landrigan PJ, Sonawane B, Butler RN, Trasande L, Callan R, Droller D. Early environmental origins of neurodegenerative disease in later life. Environ Health Perspect 2005;113:1230–3.
Langley-Evans SC. Developmental programming of health and disease. Proc Nutr Soc 2006;65:97–105.
Leasure JL, Giddabasappa A, Chaney S, Johnson JE Jr, Pothakos K, Lau YS, et al. Low-level human equivalent gestational lead exposure produces sex-specific motor and coordination abnormalities and late-onset obesity in year-old mice. Environ Health Perspect 2008;116:355–61.
Lilienthal H, Kohler K, Turfeld M, Winneke G. Persistent increases in scotopic B.-wave amplitudes after lead exposure in monkeys. Exp Eye Res 1994;59:203–9.
Lilienthal H, Lenaerts C, Winneke G, Hennekes R. Alteration of the visual evoked potential and the electroretinogram in lead-treated monkeys. Neurotoxicol Teratol 1988;10:417–22.
Ma T, Chen HH, Ho IK. Effects of chronic lead exposure on neurobehavioral function and dopaminergic neurotransmitter receptors in rats. Toxicol Lett 1999; 105:111–21.
McDonald JW, Johnston MV. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Brain Res Rev 1990;15:41–70.
Mendola P, Selevan SG, Gutter S, Rice D. Environmental factors associated with a spectrum of neurodevelopmental deficits. Ment Retard Dev Disabil Res Rev 2002;8:188–97.
Moreira EG, Vassilieff I, Vassilieff VS. Developmental lead exposure: behavioral alterations in the short and long term. Neurotoxicol Teratol 2001;23:489–95.
Nagpal AG, Brodie SE. Supranormal electroretinogram in a 10-year-old girl with lead toxicity. Doc Ophthalmol 2009;118:163–6.
Newbold RR, Padilla-Banks E, Jefferson WN, Heindell JJ. Effects of endocrine disruptors on obesity. Int J Androl 2008;31:201–8.
Newland MC, Rasmussen EB. Aging unmasks adverse effects of gestational exposure to methylmercury in rats. Neurotoxicol Teratol 2000;22:819–28.
Osman K, Pawlas K, Schütz A, Gazdzik M, Sokal JA, Vahter M. Lead exposure and hearing effects in children in Katowice, Poland. Eviron Res 1999;80:1–8.
Paule MG, Chelonis JJ, Buffalo EA, Blake DJ, Casey PH. Operant test battery performance in children: correlation with IQ. Neurotoxicol Teratol 1999;21:223–30.
Paule MG, Forrester TM, Maher MA, Cranmer JM, Allen RR. Monkey versus human performance in the NCTR Operant Test Battery. Neurotoxicol Teratol 1990;12: 503–7.
Paule MG, Li M, Allen RR, Liu F, Zou X, Hotchkiss C, et al. Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. Neurotoxicol Teratol 2011;33:220–30.
Paule MG. Validation of a behavioral test battery for monkeys. In: Buccafusco JJ, editor. Methods of behavioral analysis in neuroscience. Boca Raton: CRC Press LLC; 2001, pp. 281–94.
Reiprich P, Kilb W, Luhmann HJ. Neonatal NMDA receptor blockade disturbs neuronal migration in rat somatosensory cortex in vivo. Cereb Cortex 2005;15:349–58.
Rice D, Barrone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000; 108(Suppl. 3):511–33.
Ris MD, Dietrich KN, Succop PA, Berger OG, Bornschein RL. Early exposure to lead and neuropsychological outcome in adolescence. J Int Neuropsychol Soc 2004;10: 261–70.
Rodier PM. Chronology of neuron development: animal studies and their clinical implications. Dev Med Child Neurol 1980;22:525–45.
Rothenberg SJ, Poblano A, Schnaas L. Brainstem auditory evoked response at five years and prenatal and postnatal blood lead. Neurotoxicol Teratol 2000;22:503–10.
Rothenberg SJ, Schnaas L, Salgado-Valladares M, Casanueva E, Geller AM, Hudnell HK, et al. Increased ERG a- and b-wave amplitudes in 7- to 10-year-old children resulting from prenatal lead exposure. Invest Ophthalmol Vis Sci 2002;43:2036–44.
Scallet AC, Schmued LC, Slikker W Jr, Grunberg N, Faustino PJ, Davis H, et al. Developmental neurotoxicity of ketamine: morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. Toxicol Sci 2004;81:364–70.
Shi Q, Guo L, Patterson TA, Dial D, Li Q, Sadovova N, et al. Gene expression profiling in the developing rat brain exposed to ketamine. Neuroscience 2010;166:852–63.
Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicol Sci 2007; 98:145–58.
Stokes-Riner A, Thurston SW, Myers GJ, Duffy EM, Wallace J, Bonham M, et al. A longitudinal analysis of prenatal exposure to methylmercury and fatty acids in the Seychelles. Neurotoxicol Teratol 2011;33:325–8.
Swanson JM, Entringer S, Buss C, Wadhwa PD. Developmental origins of health and disease: environmental exposures. Semin Reprod Med 2009;27:391–402.
Taylor PD, Poston L. Developmental programming of obesity in mammals. Exp Physiol 2007;92:287–98.
Tomita H, Shibata Y, Sakurai T, Okada Y. Involvement of a protein kinase C-dependent process in long-term potentiation formation in guinea pig superior colliculus slices. Brain Res 1990;536:146–52.
Torrealba F, Valdes JL. The parietal association cortex of the rat. Biol Res 2008;41:369–77.
Vahter M, Akesson A, Liden C, Ceccatelli S, Berglund M. Gender differences in the disposition and toxicity of metals. Environ Res 2007;104:85–95.
Van den Bergh BR. Developmental programming of early brain and behaviour development and mental health: a conceptual framework. Dev Med Child Neurol 2011;53(Suppl. 4):19–23.
Verlangieri AJ. Prenatal and postnatal chronic lead intoxication and running wheel activity in the rat. Pharmacol Biochem Behav 1979;11:95–8.
Wasserman GA, Musabegovic A, Liu X, Kline J, Factor-Litvak P, Graziano JH. Lead exposure and motor functioning in 4(1/2)-year-old children: the Yugoslavia prospective study. J Pediatr 2000;137:555–61.
Weiss B, Clarkson TW, Simon W. Silent latency periods in methylmercury poisoning and in neurodegenerative disease. Environ Health Perspect 2002;110(Suppl. 5):851–4.
White RF, Palumbo CL, Yurgelun-Todd DA, Heaton KJ, Weihe P, Debes F, et al. Functional MRI approach to developmental methylmercury and polychlorinated biphenyl neurotoxicity. Neurotoxicology 2011;32:975–80.
Zou X, Patterson TA, Divine RL, Sadovova N, Zhang X, Hanig JP, et al. Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. Int J Dev Neurosci 2009a;27:727–31.
Zou X, Patterson TA, Sadovova N, Twaddle NC, Doerge DR, Zhang X, et al. Potential neurotoxicity of ketamine in the developing rat brain. Toxicol Sci 2009b;108: 149–58.