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Late effects of a perinatal exposure to a 16 PAH mixture: Increase of anxiety-related behaviours and decrease of regional brain metabolism in adult male rats

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous pollutants originated from incomplete combustion processes. Ingestion of contaminated food is the main route of exposure for humans. These molecules are able to cross the placental barrier and are also found in breast milk. Since PAHs are neurotoxic agents, the potential adverse effects of a perinatal exposure of the developing brain is a key issue for public health especially concerning PAH mixture. In this study, female rats were exposed trough diet to a mixture of 16 PAHs, at doses of 2 μg/kg/day or 200 μg/kg/day during gestation and 1.5 μg/kg/day or 150 μg/kg/day during breast-feeding period. To assess late neurotoxic effects in male offsprings, behavioural and cognitive tests were carried out and histochemical analyses using cytochrome oxidase as a cerebral metabolism marker were performed on adult animals. Results showed that anxiety-related behaviours significantly increased in exposed animals, but there was no significant alteration of motor activity and learning and memory abilities. Several brain areas of the limbic system showed a neuronal hypometabolism in exposed animals. This work highlights that exposure to PAHs at early stages of brain development can cause later troubles on behaviour and that PAHs are able to partly alter the central nervous system metabolism on adulthood.

1. Introduction

Because of its high level of organization and its very sophisticated functions, the brain is often considered as the most complex organ. Even if the nervous system of an adult is described as a well-protected system (Rodier, 1994), brain sensitivity to neurotoxic chemicals is increased when exposure occurs during early developmental stages. It has been recognized that nervous system of foetus and neonate exhibit increased sensitivity to a number of environmental chemicals due to its immaturity, and to several sensitive processes (proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination and apoptosis) occurring from foetal life to many years in childhood (Landrigan et al., 2005). It has been estimated by the American National Academy of Sciences that 3% of brain developmental disorders, such as attention-deficit alteration, mental retardation, autism spectrum disorders or learning disabilities, may be directly linked to exposure to environmental chemicals. Another 25% may result from an environmental insult occurring in combination with individual genetic predisposition (Miodovnik, 2011). Furthermore, it is important to note that early perturbations on brain development may rise to neurobehavioural disturbances expressed either in childhood or with delayed onset in adulthood (Olney, 2002). For instance evidence exists supporting the idea that lifelong susceptibility to anxiety disorders can be partly determined by environmental factors during early development (Gross and Hen, 2004).

Classified amongst persistent organic pollutants since 1998 by the Aarhus Protocol (United Nations Economic Commission for Europe, 1998), Polycyclic Aromatic Hydrocarbons (PAHs) consist of a large class of organic compounds that are constituted of two or more fused aromatic rings (Baek et al., 1991). PAHs are formed by the incomplete combustion or pyrolysis of organic matter and/or during various industrial processes. They are generally found as complex mixtures of varying composition (ATSDR, 1995). In 1984, US EPA classified 16 PAHs as priority pollutants based on their toxicity (especially carcinogenic properties), potential for human exposures, and frequency of occurrence at hazardous waste sites.

Abbreviations: B(a)P, benzo(a)pyrene; NMDA, N-methyl-D-aspartate; PAH, Polycyclic Aromatic Hydrocarbon; ROS, reactive oxygen species.

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In non-smokers, dietary intake is the major route of PAH exposure compared to inhalation (Menzie et al., 1992). The PAH contamination of food arises from environmental sources, industrial food processing and/or home food preparation (EC, 2002). PAH content in food samples and the type of food eaten by a given population are two important factors that contribute to the variations observed within exposure data reported amongst different studies. Total daily PAHs ingestion ranged from 3.70 μg/kg in United Kingdom (Dennis et al., 1983) to 12 μg/kg for an adult man in Spain (Martí-Cid et al., 2008). In the Dutch study published by de Vos et al. (1990), the PAH daily intake for an adult was estimated between 5 and 17 μg/kg. Finally it should be noticed that the PAH level of exposure *via* food consumption for a given population has increased from 8.4 to 12 μg/kg/day for an adult in 8 years (Falcó et al., 2003).

PAHs are now well established as human health hazards, especially due to their carcinogenic and mutagenic properties (IARC, 1983; Vyskocil et al., 2000). Neurotoxic effects of PAHs have not received much attention, whereas recent epidemiological and experimental data have been published (for review, see Schroeder, 2011). In humans, neurophysiological impairments were reported following exposure to several PAHs including benzo(a)pyrene (B(a)P) in plant workers (Majchrzak et al., 1990; Nie et al., 2008; Niu et al., 2009), and people living in the neighbourhood of contaminated places (Dayal et al., 1995; Kilburn and Warshaw, 1995; Dahlgren et al., 2003). In animal studies, B(a)P and fluoranthene were shown to induce different nervous system damages, including motor activity and anxiety level reductions, learning ability impairments, as well as neuromuscular and physiologic abnormalities (Saunders et al., 2002, 2003, 2006; Grova et al., 2007, 2008; Xia et al., 2011).

Currently, there is growing evidence that an early exposure to PAHs during pregnancy or breast-feeding may result in negative effects on foetal growth and child neurodevelopment. Exposure to PAHs occurs already *in utero* because these compounds are able to pass through the placenta (Madhavan and Naidu, 1995; Gladen et al., 2000; Singh et al., 2008), and during breast-feeding because they are capable of partitioning into breast milk (Del Bubba et al., 2005; Zanieri et al., 2007; Kim et al., 2008). In rodents, few studies showed some negative effects of a gestational or a lactating B(a)P exposure *in utero* and/or *via* breast milk on brain maturation and memory abilities measured through behavioural performances, glutamate NMDA receptor subunit expression, and long-term potentiation recording of pups exposed through the dam (Hood et al., 2000; Wormley et al., 2004a; Brown et al., 2007; McCallister et al., 2008; Bouayed et al., 2009a; Li et al., 2011). In addition, epidemiological data showed a significant association between a prenatal exposure to atmospheric PAHs and a developmental delay in intellectual development as well as a reduced IQ in children at respectively 3 and 5 years of age (Perera et al., 2006, 2009; Tang et al., 2008). Very recently, Perera et al. (2011) reported that a high level of exposure to PAHs during pregnancy, measured by DNA adducts in umbilical cord white blood cells, was correlated with higher symptom scores of anxious/depressed at 4.8 years and attention problems at 4.8 and 7 years. These data suggested that an early PAH exposure may adversely affect behaviour of developing children.

Thus, taken altogether, these data suggest that pollutants such as PAHs are hazardous substances for brain development. However, in animal studies the toxicity of only two PAHs, namely B(a)P and fluoranthene, has been usually assessed, leading to too restrictive conclusions in terms of human health. This demonstrates that there is a real need of data related to toxicity of chemical mixtures (Ramesh et al., 2004). Furthermore, a single and high level of exposure is often used within these studies, which does not reflect a real environmental dietary exposure, which is rather characterized by repeated and low doses of chemicals mixture over a longer period.

Consequently, the aim of the present study was to evaluate in rat pups the late consequences in terms of behaviour and brain metabolism of an perinatal exposure to a PAH mixture administrated through the diet to the dam during both gestational and lactational periods.

2. Materials and methods

2.1. PAH mixture

The 16 PAHs were purchased from Sigma–Aldrich (St. Quentin Fallavier, France) as a powder for each compound (purity between 96% and 99%). Each PAH was weighed separately and the 16 PAHs were mixed into oil (Isio 4, Lesieur, Asnières sur Seine, France). To dissolve them, the mixture was sonicated (55 Hz) for 2 h at room temperature. The composition of the mixture was defined in order to respect the proportion of each PAH described in studies focusing on daily PAH dietary intake (EC, 2002; Martí-Cid et al., 2008; EFSA, 2008), as follow (w/w): phenanthrene (25%), naphthalene (17%), fluoranthene (11.5%), acenaphthene (10%), pyrene (8.5%), indeno[1,2,3,c,d]-pyrene (6%), chrysene (4%), anthracene (3.5%), benz[a]pyrene (2.5%), benzo[b]fluoranthene (2.5%), fluorene (2.5%), benz[a]anthracene (2%), benzo[k]fluoranthene (2%), acenaphthylene (1.5%), benzo[g,h,i]perylene (1%), dibenz[a,h]anthracene (0.5%).

2.2. Animals

Twenty female Wistar rats, weighing 175–199 g, were obtained from Harlan Laboratories (France). Upon arrival, the females were housed at 2 per cage. Animals were maintained under a reverse light cycle (lights on at 7 p.m., lights off at 7 a.m.), at a constant temperature (22 ± 2 °C) and a relative humidity of 55 ± 10%. Rats were given free access to food and water. After a 1-week period for acclimatization (Fig. 1), females underwent the exposure protocol (see Section 2.3 for details) each morning during a week. Then, females were mated with breeding males (2 females with 1 male) over day, and were examined the following evening by vaginal smear to assess successful mating. When occurring, the female was removed from the cage of the male and was housed individually. This day was considered as the day 0 of gestation (GD0). Then, female exposure to PAHs occurred daily from GD1 to postnatal day 21 (PND21). The day of birth was considered as PND0. On PND0 litters were reduced to 10 pups when possible, as recommended by the 426 OECD guideline (2007) in order to prevent the effects of litter size on pup development. Behavioural tests were performed at the adult stage (from PND60). On PND60 one male per litter was sacrificed, and the brain was removed for histological analyses.

All these experiments on animals were performed in respect with the rules provided by the European Union (Directive 2010/63/EU).

2.3. Doses and protocol of exposure

Two different doses were used. The first one, mentioned as Dose 1 in the present study, was of 2 μg/kg/day during gestation and 1.5 μg/kg/day during lactation. This lowest dose was calculated to model the quantity of PAHs daily ingested by a woman during pregnancy (9 months) and period of breast-feeding considered by the World Health Organization (6 months). The second dose, mentioned as Dose 2 in this study, corresponded to a 100-fold higher dose, *i.e.* 200 μg/kg/day during gestation and 150 μg/kg/day during lactation (Dose 2). This highest dose was considered to be toxic, as recommended by the 426 OECD guideline (2007) for assessment of the developmental neurotoxicity of chemical compounds. The PAH exposure started on GD1 and was stopped on PND21. To exclude the participation of a litter effect in the final results, the experiments were performed on at least 6 different litters for each group. Amongst the 20 females, 6 were allocated to the control group (fed with food added with Isio 4 oil only), 7 were exposed to the lowest dose of PAHs (Dose 1), and 7 were exposed to the highest one (Dose 2). The PAH exposure was made by daily feeding each female with 1 g of contaminated rat food. The contaminated food was prepared each morning before 9 a.m. by mixing 1 g of powder rat food usually used with water and sweet syrup in order to obtain a homogenous pastry. Then the appropriate volume of oil or the PAH solution was added according to the weight of each female. The pastry was presented as a pellet to animals. Each female was isolated in a small cage without sawdust and was given a 5 min meal. During the whole experimental period, all pellets were completely eaten by each female and no residual was found in the cage. The use of this protocol of exposure allowed the authors of the study to control daily the complete ingestion of contaminated food, and to guarantee the complete PAH intake.

2.4. Behavioural testing

From PND60, behavioural tests were performed on the same 2 males from each litter in order to assess the locomotor activity in the open-field, the level of anxiety in the elevated-plus maze, the short-term memory in the Y-maze, and the spatial memory performances in the eight-arm maze. For the open-field, the elevated-plus maze and the Y-maze, one test was carried out per day, within the same week. The eight-arm maze was performed over the two following weeks during 14 consecutive