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Article *in* Toxicology Letters · December 2015

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Toxicology Letters 245 (2016) 7–14

Contents lists available at ScienceDirect

Toxicology Letters

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No evidence for oxidative stress in the cerebellar tissues or cells of juvenile male mice exposed via lactation to the 6 non-dioxin-like PCBs at levels below the regulatory safe limits for humans

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HIGHLIGHTS

- We exposed dam mice to ∑6 NDL-PCBs (0, 1, 10 and 100 ng/kg b.w./day; PNDs 0–14).
- Oxidative status was evaluated in the cerebellum of PCB lactationally exposed pups at PND 14.
- No differences in ROS production, oxidative stress-related gene expressions or protein levels were shown between groups.
- Lactational exposure to ∑6 NDL-PCBs did not induce an oxidative damage in male mice at brain growth spurt.

ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form 15 December 2015
Accepted 18 December 2015
Available online 24 December 2015

Keywords:
Non-dioxin-like polychlorinated biphenyls
Lactational exposure
Central nervous system
Oxidative stress
Development
Neurons
Glial cells

ABSTRACT

The developing central nervous system is particularly vulnerable to environmental contaminants such as non-dioxin-like polychlorinated biphenyls (NDL-PCBs). This study investigated the potential oxidative effects in mice pups exposed via lactation to the sum of the six indicator NDL-PCBs (∑6 NDL-PCBs) at 0, 1, 10 and 100 ng/kg per 14 days, constituting levels below the guidance values fixed by French food safety agencies for humans at 10 ng/kg body weight per day. For this purpose, the oxidative status was assessed by flow cytometry via dichloro-dihydro-fluorescein diacetate in the cerebellum of juvenile male offspring mice during brain growth spurt [postnatal day (PND) 14]. No significant differences were found in the levels of reactive oxygen species in the cerebellar neurons or glial cells (astrocytes, oligodendrocytes and microglia) of lactationally exposed male mice at PND 14 ($p > 0.05$). Concordantly, oxidative-stress related gene expression was measured by qPCR for catalase, copper zinc superoxide dismutase 1, glyoxalase 1, glutathione peroxidase 1, and glutathione reductase 1, in the cerebellum at PND 14 appeared unaffected, as also verified at the protein level by immunoblots. Moreover, transcriptomic data from our previous work have not shown differences in the mRNA expressions of genes belonging to GO terms involved in oxidative stress in neurons of male mice exposed to ∑6 NDL-PCBs compared to controls; except for glyoxalase 1 which was downregulated in neurons isolated from exposed group compared to controls. Our findings suggest that lactational exposure to NDL-PCBs at environmental relevant concentrations may not cause significant oxidative effect on juvenile cerebellum.

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1. Introduction

Non-dioxin-like polychlorinated biphenyls (NDL-PCBs) are persistent organic chemicals that accumulate in the environment and biological tissues. Once stored in adipose tissues, they are

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http://dx.doi.org/10.1016/j.toxlet.2015.12.003
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transferred from the mother to the offspring via breastfeeding (Vigh et al., 2013). This transfer may contribute to 5–10% of the total body burden of NDL-PCBs observed at adult age (Afssa-Avis, 2015), and results in critical vulnerability during neonatal life, altering maturation processes in the developing central nervous system (Grandjean and Landrigan, 2014). In humans, rapid development in the brain spans from the third trimester of gestation and ends by the third year of life while in mice, it occurs within the first month of life, peaking at 10 days of age (Dobbing, 1975). During this period often called the "brain growth spurt"., the developing brain is more vulnerable to toxic insults than the adult brain. NDL-PCBs can alter neuron functioning, resulting in long-lasting changes to the brain (Elnar et al., 2015b; Kodavanti, 2005; Pessah et al., 2010).

We have recently reported persistent anxiety-like behavior in male mice lactationally exposed to environmentally plausible levels (1, 10 and 100 ng/kg/day) of the sum of the six indicator NDL-PCBs (PCBs 28, 52, 101, 138, 153 and 180; Elnar et al., 2012). These 6 NDL-PCBs, representing the most predominant PCB congeners in contaminated fish matrices (EFSA, 2012, 2010), have also shown to increase the expression of ryanodine receptor 3 (RyR3) in the cerebellum of the juvenile mice (Elnar et al., 2012), with a p53-dependent response to cellular stress and a decrease in the expression of proteins involved in the transmission of electrical signals in neurons of the exposed mice (Elnar et al., 2015a).

In general, it has been shown that the developing brain has lower levels of antioxidants (Tian et al., 1998) and that the antioxidant system in young animals is less efficient than in adults. Thus, a decrease in antioxidant enzyme activity could be expected following the exposure to environmental insults, such as NDL-PCBs (Vicente et al., 2004). In addition, many studies have shown the well-known pro-oxidant effect of PCB commercial mixtures such as Aroclor 1254 (Venkataraman et al., 2010), whose constitution is relatively high in NDL-PCB congeners (Frame et al., 1996a,b). Given the link between oxidative stress and anxiety (Bouayed et al., 2009), we hypothesized that exposure to NDL-PCBs could also have a putative mechanism by which it may disrupt the oxidative status of neurons, at a period where the central nervous system is particularly susceptible to free radical related oxidative damage (Buonocore et al., 2001). This could be responsible for the anxiety-like behavior observed at more advanced life stages.

Thus, in the present investigation, we examined a new set of F1 male mice whose mothers were lactationally exposed to $\sum$6 NDL-PCBs and their controls, in order to evaluate the level of intracellular oxygen-derived species in neurons and glial cells. In addition, gene expression and protein changes of stress-related enzymes, important in the detoxification of oxygen radicals such as catalase (Cat), copper-zinc superoxide dismutase 1 (Cu/ZnSod1), glyoxalase 1 (Glo1), glutathione peroxidase 1 (GPx1), and glutathione reductase 1 (Gsr1), were investigated in the cerebellar tissues of the juvenile male mice at brain growth spurt, i.e. at postnatal day (PND) 14, the end of the exclusive lactational exposure.

2. Materials and methods

2.1. Chemicals

The fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) was purchased from Sigma–Aldrich (Saint Louis, MO, USA). The congeners 2,4,4′-trichlorobiphenyl (PCB 28; batch no. SZE6317X; 99.9% purity), 2,2′,5,5′-tetrachlorobiphenyl (PCB 52; batch no. SZE6234X; 99.6% purity), 2,2′,4,5,5′-pentachlorobiphenyl (PCB 101; batch no. SZE6298X; 99.9% purity), 2,2′,3,4,4′,5′-hexachlorobiphenyl (PCB 138; batch no. SZE7089X; 99.6% purity), 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153; batch no. SZE9271X; 99.5% purity) and 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (PCB 180; batch no. SZE9105X; 99.5% purity) were obtained from Sigma–Aldrich Co. (St. Quentin Fallavier, France, purity >99%). Hank's balanced salts solution (HBSS) was purchased from Sigma–Aldrich. MACS buffer and neural tissue dissociation kit (papain and trypsin) were acquired from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.2. Animals and treatment

In this study, we used twenty female and twenty male Swiss Albino mice (OF1, Janvier; France), aged nine weeks, and weighing between 30 and 40 g. Animals were housed with five mice/cage/sex under a 12-h light/12-h dark schedule (lights on at 8:00 pm), with free access to water and food (SDS Dietex; France). Mice were maintained at a constant temperature (22 ± 2°C) and a relative humidity of 55 ± 10%. Following an acclimatization period of one week, female mice were mated overnight and examined the following morning for copulatory plugs. The fertilization rate was 80% (16 of 20). Gravid females were individually housed and were assigned to experimental groups by stratified randomization ($n = 4$ dams/group). A cotton nest square as a source of nesting material was supplied one week before the delivery. The day of parturition was considered as PND 0. At this date, litters were culled to ten pups with equal sex ratio, when possible. The dams' weight was recorded daily to adjust treatment levels.

Mice dams received 0, 1, 10 and 100 ng/kg b.w./day of a mixture of pure molecules of $\sum$6 NDL-PCBs [PCBs 28 (2%), 52 (6%), 101 (12%), 138 (32%), 153 (37%), and 180 (11%)] in rapeseed oil (10 ml/kg b.w.) by oral gavage, starting from parturition (PND 0) until the end of exclusive lactation (PND 14). The preparation of the stock solution of $\sum$6 NDL-PCBs and the composition of the 6 NDL congeners (PCBs 28, 52, 101, 138, 153 and 180) in the mixture were detailed previously (Elnar et al., 2012).

On PND 14, only two males of the 10 pups were randomly selected from each litter for analyses, anaesthetized with halothane, and then sacrificed, taken into account the litter as unit of variance in this type of study and to avoid the litter effect (Holson et al., 2008; OECD, 2007).

One male pup per litter was used for cytometric analysis of isolated cerebellar cells ($n = 4$/group), and the second male pup from the same litter was used for detection of mRNA and protein expression of enzymes from the whole cerebellar tissues ($n = 4$/group). Cerebellar tissues were homogenized in RLT buffer [RNeasy mini kit (Qiagen; Leusden, The Netherlands)] by vortex using two sterile metallic balls per sample (that had been cooled with liquid nitrogen). Tissues were snapped frozen in liquid nitrogen, and then stored at −80°C until RNA and protein extraction and analysis. All animal use and care procedures were performed in accordance with the Directive 2010/63/EU, and were approved by the local research ethics committee of the University of Lorraine (CELMEA-2013–0010).

2.3. Assessment of oxidative status in cerebellar neurons and glial cells

2.3.1. Neuron and glial cell dissociation

On PND 14, the cerebellum of male mice was excised from the brain in order to isolate neurons or glial cells, according to the manufacturer's protocol (Miltenyi Biotec). In brief, the cerebellar tissue was removed, homogenized using a potter in 1 ml of cold HBSS. The cell suspensions were centrifuged ($300 \times g$, 4°C, 2 min), and the supernatants were discarded. The tissues were dissociated by incubation with papain (for neuron and oligodendrocyte collection) or with trypsin (for astrocyte and microglia isolation) as described in the neural tissue dissociation kit. Cells were manually triturated, and were passed through a 30 μm nylon mesh (pre-separation filters, Miltenyi Biotec) to remove cell clumps. After centrifugation of the solutions ($300 \times g$, 4°C, 10 min), the supernatants were

discarded. Pellets were resuspended in 80 μl of MACS buffer and 20 μl of cluster differentiation (CD) 90.2 microbeads (Miltenyi Biotec; Bergisch Gladbach, Germany) were added. The solutions were mixed, and incubated at 4°C for 15 min. The pellets were washed with 2 ml of MACS buffer, centrifuged (300 × *g*, 4°C, 10 min), and supernatants were discarded. The cells were resuspended in 500 μl of MACS buffer, and the cell suspension was separated on a MiniMACS MS column by adding 1 ml of buffer, according to the manufacturer's instructions (Miltenyi, Biotec).

The magnetically labeled cells were retained on the column and fractions were consecutively flushed out with the magnetically labeled cells by firmly applying the plunger supplied with the column. Thus, CD 90.2, which is expressed in neurons, was magnetically separated from the other cells in the mouse brain using anti-CD 90.2 microbeads in the MACS® column separator (Miltenyi Biotec). The same principle was applied to astrocytes, oligodendrocytes and microglia using anti-GLAST, anti-O4 and CD 11b microbeads, respectively (Miltenyi Biotec). Neurons or glial fractions were collected, washed with 1 ml of MACS buffer, and centrifuged (300 × *g*, 4°C, 10 min). Supernatants were discarded, and pellets containing 10^7 viable cells per brain were resuspended in 1 ml of MACS buffer. 80 μl of the cells were mixed with 5 μl of DCFH-DA (50 μM) and incubated at 37°C for 15 min in order to quantify reactive oxygen species (ROS) by flow cytometry. Negative controls (without DCFH-DA) and positive controls [with H_2O_2 (10 μM)] were included in the experiment to check the validation of the experiment and fluorescent probe detection.

2.3.2. Flow cytometric measurements

The samples were analyzed using a FACScan using Cell Quest software (Becton-Dickinson, France), similar as published earlier (Belhaj et al., 2013). Cells were gated according to their size by forward scatter and granularity by side scatter. DCFH-DA/FITC was excited by a 488 nm argon laser, and emissions were collected at 525 nm. Cells were sorted into cold HBSS.

2.4. Microarray analyses

This section was detailed previously (Elnar et al., 2015a).

2.5. RNA extraction and real time qPCR

Total RNA was extracted from 60 mg of frozen mice cerebellum (PND 14) using the RNeasy mini kit (Qiagen; Leusden, The Netherlands), including DNase treatment according to the manufacturer's instructions. Quality control was performed with the RNA 6000 Nano assay using a 2100 Bioanalyzer (Agilent Technologies; Massy, France). RNA samples with an RNA integrity number lower than seven were excluded from the experiment. RNA purity and concentrations were assessed measuring the absorbance at 230 nm, 260 nm, and 280 nm, employing a Nanodrop ND-1000 spectrophotometer (Thermo Scientific; Villebon-sur-Yvette, France).

We followed five target genes: Cat, Cu/ZnSod1, Glo1, GPx1, and Gsr1, whose expressions levels were compared to those of six housekeeping genes as controls [actin-β (Actb), aryl hydrocarbon receptor interacting protein (Aip), CXXC finger protein 1 (Cxxc1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mitochondrial ribosomal protein L48 (Mrpl48), and RNA polymerase II (RpolyII)]. Primer sequences were selected using the Primer-BLAST Genbank, and are included in the Supplementary Table.

Reverse transcription was performed using 1 μg of RNA in a final volume of 20 μl including 0.5 μl of random primers (3 μg/μl; Invitrogen, Carlsbad, NM, USA), 1 μl of 10 mM dNTP mix, in RNase-free water (Invitrogen). After denaturation of RNA samples at 65°C for 5 min, 4 μl of buffer (5×), 2 μl of 0.1 mM DTT, 1 μl of RNase OUT (Invitrogen), and 1 μl of superscript II reverse transcriptase (Invitrogen; Carlsbad, NM, USA) were added. Samples were homogenized and were transcribed in a thermal cycler (Biometra, Professional Basic Thermocycler 96 wells, Göttingen, Germany), according to the following conditions: 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min.

PCR was performed using the MESA GREEN Low ROX real-time PCR kits (Eurogentec; Liège, Belgium) with the following final concentrations in a 25 μl final volume: 1 × Master Mix, 100 nM forward and reverse primers, 0.4 ng/μl cDNA. The mix was placed in a 7500 Fast Real-Time PCR system (Applied Biosystems; Foster City, CA, USA). The thermal cycling conditions were: initial 5 min denaturation at 95°C, followed by 50 cycles of 15 s at 95°C, 1 min at 60°C, and a final dissociation step. The primer specificity was determined based on the presence of a single peak in the melting curve and the PCR efficiency was assessed using a decreasing five-fold dilution (25–0.04 ng and no cDNA; Supplementary Table). Quantitative PCR (qPCR) assay was in agreement with the minimum information for publication of real-time qPCR experiments (MIQE) guidelines (Bustin et al., 2009). The relative expression was calculated using multiple reference genes, and gene-specific PCR efficiency; the most stable housekeeping genes, namely Actb, Mrpl48, and Cxxc1 were selected using the geNorm software (Vandesompele et al., 2002). All calculations were performed using the qbase plus software (http://www.biogazelle.com).

2.6. Protein extraction and Western immunoblot analyses

Cerebellar tissues were solubilized in RIPA lysis buffer [136 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% NP40, 0.5% DOC, 0.1% SDS, 0.05 mM PMSF, 0.1 mM Na_3VO_4, and Protease Inhibitor Cocktail, (Roche Applied Science, Meylan, France)]. They were incubated on ice for 10 min. After homogenization by pipetting, tissues were disrupted by three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. Samples were centrifuged at 9000 × *g* for 30 min at 4°C, supernatants were recovered, aliquoted and stored at −80°C until further analyses. The supernatant protein concentrations were determined using the Pierce BCA assay kit (Thermo Fisher Scientific, Saint-Herblain, France). Aliquots containing 15 μg of proteins were prepared for each sample, mixed with an equal volume of 2 × Laemmli buffer and denatured by heating at 100°C for 10 min.

After protein separation in SDS-PAGE, protein levels were investigated by immunoblotting using anti-catalase (Cat; 1:500; ab125688), anti-glutathione reductase (Gsr1; 1:500; ab16801), anti-superoxide dismutase (Sod1; 1:400; ab88404), and anti-actin β (Actb; 1:500; ab13822) antibodies (abcam, Cambridge, England). Immunoblots were probed with the corresponding conjugated secondary antibodies (1:1000; Cell Signaling), using the enhanced chemiluminescence detection protocol (ECL Kit, Amersham Biotech, Velizy-Villacoublay, France). Immunoblots were digitized using the Fusion FX5 image acquisition system (Vilber Lourmat, Collégien, France), and protein signals were quantified with Image J densitometry software. Relative protein levels were normalized to Actb signal intensities.

2.7. Statistical analyses

The distribution was checked for each variable using Kurtosis and Skewness tests. Since no Gaussian distribution was found, non-parametric statistics using the Kruskal–Wallis test were conducted followed by the Mann–Whitney *U*-test for *post hoc* analyses between PCB exposure groups and controls. Results were reported as median (IQR), and significance was set at $p < 0.05$

(2-sided). Statistical analyses were carried out using the Statview® 4.5 software (Abacus Concepts, Inc.; New Jersey, USA). Origin 8.5 software was used to create the figures (Origin 8.5G Software, OriginLabs, Northampton, MA, USA). Concerning our microarray data, statistical analyses were performed as described previously (Elnar et al., 2015a).

3. Results

3.1. Effects of ∑6 NDL-PCBs on oxidative status

ROS levels were measured in various cerebellar cell types of male mice exposed to ∑6 NDL-PCBs and compared to control animals. As concluded from statistical analysis using the Kruskal Wallis test, no significant changes could be noticed between NDL-PCB mice at any concentration in the neurons ($H = 3.51$, $p = 0.32$), in the microglial cells ($H = 0.46$, $p = 0.93$), in the astrocytes ($H = 0.88$, $p = 0.83$) or in the oligodendrocytes ($H = 3.46$, $p = 0.33$) on PND 14 (Fig. 1). These observations suggest that the mix of 6 NDL-PCBs did not induce significant oxidative stress.

3.2. Effects of ∑6 NDL-PCBs on oxidative stress-related mRNA expression

Expression levels were estimated after RT-qPCR analysis of the RNA fraction extracted from the cerebellar tissues. The Kruskal–Wallis test revealed no significant differences between mice exposed to ∑6 NDL-PCBs and control mice in the mRNA expressions of Cat ($H = 2.96$, $p = 0.40$), Cu/ZnSod1 ($H = 1.30$, $p = 0.73$); Glo1 ($H = 3.51$, $p = 0.32$); GPx1 ($H = 4.60$, $p = 0.21$), and Gsr1 ($H = 0.11$, $p = 0.99$), although a high inter-individual variability could be observed within the different groups of mice, especially the mice exposed via lactation to NDL-PCBs at the dose of 100 ng/kg/day (Fig. 2). These data suggest that the mixture of 6 NDL-PCBs did not induce the gene expression of these essential antioxidant enzymes.

In addition, our results focusing on the transcriptomic profile of cerebellar neurons isolated from PND 14 male mice exposed via lactation to ∑6 NDL-PCBs at 10 ng/kg/day, have shown that the Gene Ontology terms involved in oxidative stress markers were not changed between controls and exposed groups, except for Glo1, which was downregulated in neurons isolated from PCB-exposed mice compared to controls (Table 1). When focusing on the same genes' expression in cerebellar tissues, we can corroborate the finding by qPCR, as no significant changes were found in the relative mRNA expression of Cat, Cu/ZnSod1, Glo1, GPx1, and Gsr1 ($p > 0.05$; Fig. 2).

3.3. Effects of ∑6 NDL-PCBs on oxidative stress-related protein levels

Protein levels were estimated by immunoblot analysis of cerebellar crude extracts and led us to observe similar profiles than the mRNA levels of the corresponding genes. Statistical analyses revealed non-significant differences between the different groups of NDL-PCB-treated mice when comparing the relative protein levels (normalized to Actb) of Cat, Gsr1 and Sod1 to those in control animals ($H = 4.53$, $p = 0.21$; $H = 0.29$, $p = 0.73$; $H = 0.87$, $p = 0.83$, respectively; Fig. 3). These data were in agreement with those above, leading to the idea that lactational exposure to NDL-PCBs did not appear to generate pro-oxidative conditions, at least in cerebellum, as observed on PND 14. This suggests that these tissues did not suffer from increased ROS production that would have required appropriate induction of antioxidant enzymes in the cerebellar cells.

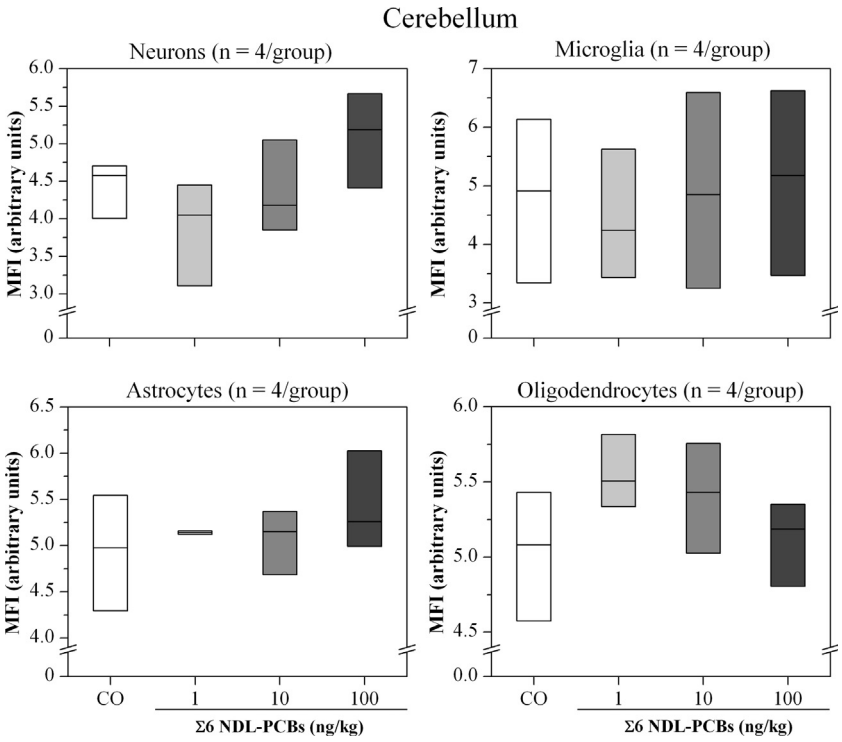

Fig. 1. Effects of ∑6 NDL-PCBs on oxidative status. The effect of lactational exposure to ∑6 NDL-PCBs at different concentrations (1, 10 and 100 ng/kg/day) compared to controls (CO) on reactive oxygen species production (mean fluorescence intensity: MFI) in the cerebellar neurons, microglia, astrocytes, and oligodendrocytes of juvenile male mice on postnatal day 14, using flow cytometry ($n = 4$/group). Box plot diagrams of the medians are shown with the box depicting the range of values from the 25th percentile (lower bar) to the 75th percentile (upper bar). No significant differences were observed between between groups exposed to ∑6 NDL-PCBs at the three concentrations and control groups (Kruskal–Wallis test; $p > 0.05$).

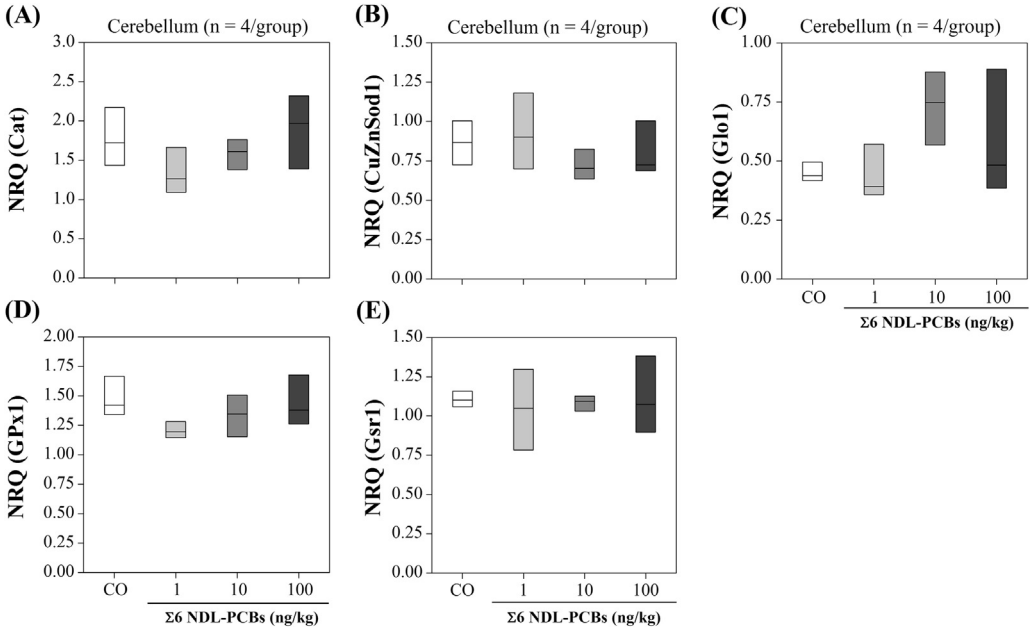

Fig. 2. Effects of ∑6 NDL-PCBs on oxidative stress-related mRNA expression. The effect of lactational exposure to ∑6 NDL-PCBs at different concentrations (1, 10 and 100 ng/kg/day) compared to controls (CO) on the mRNA expressions of catalase (Cat), copper zinc superoxide dismutase 1 (Cu/ZnSod1), glyoxalase 1 (Glo1), glutathione peroxidase 1 (GPx1), and glutathione reductase 1 (Gsr1; A, B, C, D, and E) in the cerebellum of juvenile male mice at postnatal day 14. The mRNA levels were measured by qPCR and normalized to Actb, Mrpl48, and Cxxc1 (normalized relative quantity: NRQ; $n = 4$/group). Box plot diagrams of the medians are shown with the box depicting the range of values from the 25th percentile (lower bar) to the 75th percentile (upper bar). No significant differences were observed between between groups exposed to ∑6 NDL-PCBs at the three concentrations and control groups (Kruskal–Wallis test; $p > 0.05$).

4. Discussion

In humans, the range of exposure to the ∑6 NDL-PCBs has been estimated to be 10–45 ng/kg b. w. per day (EFSA, 2005). There are no experimental studies evaluating the potential neurotoxic responses induced by lactational exposure to such or lower levels than those indicated by regulatory food agencies. Therefore, we selected this lower, environmentally plausible concentration range for our trials. In the present study, the cerebellum of male mice was chosen as a brain region of interest as it seems to be particularly affected by lactational exposure to NDL-PCBs (Elnar et al., 2015a,b, 2012). Due to the biomagnification and lipophilic properties of those xenobiotics, most of the exposure to PCBs occurs via postnatal lactation (Lee et al., 2007). Here, the time-course of the kinetics of PCB transfer from the dam to the offspring could play an important role in the toxicological effects observed in the exposed animals' central nervous system (Buonocore et al., 2001).

However, possibly due to the rather low doses administered in the present work, we have shown here that lactational exposure to ∑6 NDL-PCBs did not induce measurable oxidative stress at PND 14 in the cerebellum of the F1 juvenile male mice. In contrast to lactational exposure, the long term effects of gestational and lactational exposure to PCBs on brain development and behavior are increasingly well documented (Johansen et al., 2014; Meerts et al., 2004). In this context, we have previously shown that lactational exposure to ∑6 NDL-PCBs at the same levels could have long-lasting effects in the young and adult mice, such as elevated levels of anxiety, especially in male mice offspring (Elnar et al., 2012) with a susceptibility to neuronal stress at a mature age (Elnar et al., 2015b). In our transcriptomic analyses conducted under the same conditions of exposure in male mice from the same litters, we have shown an altered gene expression patterns related to DNA damage in exposed male mice compared to controls (Elnar et al., 2015a). Unpublished data from the same work and integrated in this study, have shown no differential transcriptomic signatures for oxidative stress in the neurons of the animals at PND 14, as the mRNA gene expression pattern of the neuronal anti-oxidant enzymes of male pups lactationally exposed to ∑6 NDL-PCBs (10 ng/kg/day) was not changed compared to the controls, except for Glo1, which was downregulated in the treated group.

4.1. NDL-PCB effects on ROS formation

Various *in vivo* and *in vitro* studies have highlighted the involvement of NDL-PCBs in the altered concentrations of intracellular calcium (Ca^{2+}), neurotransmission, gene expression, the induction of ROS formation and reduced cell viability in several brain regions of the animals (Castoldi et al., 2006; Elnar et al., 2015a,b; Fonnum and Mariussen, 2009; Langeveld et al., 2012; Pessah et al., 2010; Westerink, 2014, 2006). For example, a

Table 1
Regulation of genes related to oxidative stress in cerebellar neurons isolated from postnatal day 14 mice following lactational exposure to ∑6 NDL-PCBs (10 ng/kg/day; n = 3/group) as determined by microarray analyses.

Genbank accession number	Description	Gene Symbol	Regulation	Fold Change	*p*
NM_009804	*Mus musculus* catalase	Cat	–	0.94	0.04
NM_011434	*Mus musculus* copper zinc superoxide dismutase 1	Cu/ZnSod1	–	1.03	0.01
NM_001113560	*Mus musculus* glyoxalase 1	Glo1	Downregulated	−2.90[a]	0.02
NM_008160	*Mus musculus* glutathione peroxidase 1	GPx1	–	1.33	0.03
NM_010344	*Mus musculus* reductase 1	Gsr1	–	1.28	0.04

[a] The mRNA expression of Glo1 was downregulated in neurons of PND 14 male mice exposed to ∑6 NDL-PCBs at 10 ng/kg/day compared to controls (*t*-test; FC > 2, $p < 0.05$).

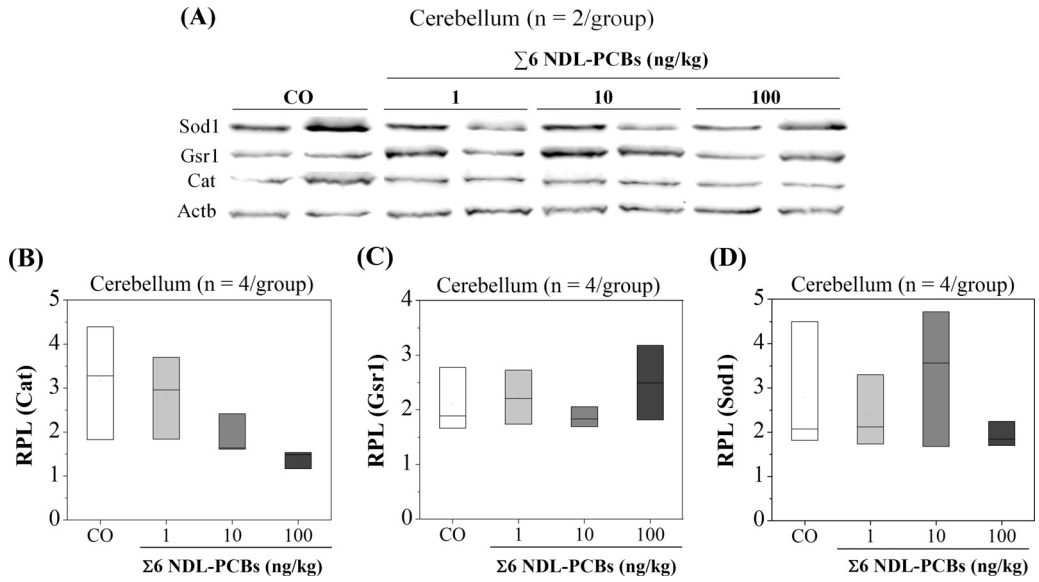

Fig. 3. Effects of ∑6 NDL-PCBs on oxidative stress-related protein levels. Cerebellar tissues were isolated from juvenile male mice at postnatal day 14 following lactational exposure to ∑6 NDL-PCBs at different concentrations (1, 10 and 100 ng/kg/day), and compared to controls (CO). (A) Two representative samples were presented from each group, allowing to measure the levels of Cat, Gsr1 and Sod1 in each sample after normalization to actin-β (Actb) used as reference protein. The median relative protein levels (n = 4/group) of (B) catalase (Cat), (C) glutathione reductase (Gsr1), and (D) superoxide dismutase (Sod1) were calculated and represented by box plot diagrams depicting the range of values from the 25th percentile (lower bar) to the 75th percentile (upper bar). Kruskal Wallis test revealed no significant differences between groups exposed to either ∑6 NDL-PCB concentration and the control group (p > 0.05).

significant increase in the basal concentration of Ca^{2+}, and an inhibition of voltage-dependent calcium channel was observed in PC12 cells exposed to PCBs 53 and 95 (0.1 and 1 μM) and to PCBs 47, 51, 52 and 104 (1 μM), while hexa- and hepta-chlorinated congeners generally did not affect the depolarization of calcium channel (Ca^{2+}; Langeveld et al., 2012). It has been also shown that the activity of PCBs was inhibited by antagonists of RyR and α-tocopherol in the hippocampal neurons in rats, indicating a link between Aroclor 1254/PCB 47 exposure and the induction of Ca^{2+}, apoptosis and oxidative stress (Howard et al., 2003). *In vitro* studies have suggested that NDL-PCB congeners, including PCB 153 (25 and 50 μM), induced oxidative stress in cultured cerebellar granule cells of rats by initiating the release of calcium from intracellular storage sites via RyR (Mariussen et al., 2002). Moreover, an increased ROS production was observed in synaptosomes of young rats following exposure to individual NDL congeners (PCBs 1, 4, 11 and 19) at 12.5 μM (Voie and Fonnum, 2000), and in cerebellar granule cells exposed to PCB 153 at 25 and 50 μM (Mariussen et al., 2002). Similarly, a reduction of cell viability has been reported in the cerebellar granule cells of juvenile rats exposed to PCB 153 and Aroclor 1254 at 6.25, 12.5, 25 and 50 μM *in vitro* (Mariussen et al., 2002). In contrast, other studies did not find an increased rate of cell death in the central nervous system of mice at gestational day 21 with PCB 153 (3–200 μM) (Costa et al., 2007), and perinatal exposure to Aroclor 1254 (6 mg/kg) did not affect the structure of Purkinje cells in PND 21 rats (Roegge et al., 2006).

Convergent with our finding of absence of altered oxidative status in the neurons or glial cells of male mice at PND 14, the present study shows that mice lactationally exposed to ∑6 NDL-PCBs have no differences in oxidative stress-related gene or protein expression of Cat, Glo1, GPx1, Gsr1, and Cu/ZnSod1 in the cerebellar tissues of mice.

4.2. Effects of NDL-PCBs on gene and protein expression

Few research so far has focused on the effect of the developmental exposure to PCB commercial mixtures (such as Aroclor 1254) on the gene expression and enzymatic activity of antioxidant enzymes in animal brains (Lee et al., 2012, 2006; Lee and Opanashuk, 2004; Yang and Lein, 2010), and relatively little is known about the effect of NDL-PCB exposure. Nevertheless, it must be taken into account that commercial mixtures such as Aroclor 1254 contain DL as well as NDL compounds, with high amounts of the six indicators investigated in the present study (i.e., PCBs 28, 52, 101, 138, 153, and 180; Frame et al., 1996a,b). Thus, it is important to have a look on the pro-oxidant effects of Aroclor 1254 in the brain even though the exposure date and/or dose are different from this study. Most of the previous studies that investigate the mechanisms of PCB toxicity have focused on planar, dioxin-like congeners or commercial mixtures such as Aroclors or NDL-PCBs with doses being 10 to 1000 fold higher than the levels used in this study. Therefore, it is difficult to compare our findings with previous studies, due to variations of the administration route, levels and time of exposure, and composition of PCB congener profile (single *vs.* complex mixtures), in addition to other alterations of laboratory parameters (*in vivo* or *in vitro* assessments, measurement at gene or protein level, etc.).

In earlier studies, it has been shown that the developmental exposure to Aroclor 1254 significantly decreased the enzymatic activity of Sod, Cu/ZnSod, MnSod, and GPx and the mRNA expression of Cu/ZnSod and GPx4 in several brain regions of exposed rats (Venkataraman et al., 2010). Other studies have provided evidence that PCB exposure may also result in changes in enzymatic activity and oxidative stress, with the most prominent effect observed in the cerebellum of PND 3 gestationally and lactationally exposed rats to Aroclor 1254 at 1 mg/kg/day (Yang and Lein, 2010). Intraperitoneal exposure of rats to Aroclor 1254 (2 mg/kg) for a period of thirty days caused reduced levels of Sod, Cat, Gpx, and Gsr in the cerebellum, cortex, hippocampus (Venkataraman et al., 2007), and hypothalamus (Muthuvel et al., 2006). A reduction in the levels of hydrogen peroxide and an increase in lipid peroxidation, as well as depletion of glutathione levels, were also found in Aroclor 1254-exposed rats (Venkataraman et al., 2007). Moreover, significant production of ROS levels with

dopaminergic cell loss (Lee et al., 2006), as well as changes in the antioxidant enzyme levels and protein expression of MnSod and Cu/ZnSod were shown in the striatum and cerebellum of mice subchronically and orally exposed to Aroclor 1254 for 21 days (6, 12, 25 mg/kg; Lee et al., 2012).

In conclusion, though lactational exposure to a realistic mixture of NDL-PCB indicators, at concentrations recognized lower than guidance values recommended by French food safety agencies, led to late persistent effects in mice including increase of anxiety (Elnar et al., 2012) and higher sensitivity to neuronal stress as we recently reported (Elnar et al., 2015b), it seems from our data in the present study that this prolonged postnatal exposure (during 14 days) did not induce oxidative stress in the juvenile brain, as measured at PND 14 via ROS measurements as well as gene and protein expression. However, the possibility that transient changes in gene expression could have occurred before PND14 cannot be ruled out. These observations suggest that the underlying cellular mechanisms of NDL-PCB toxicity still remain unclear, and more research is warranted to clarify their effects on cellular mechanisms observed in previous studies.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

Fabian Marin is thanked for his dedicated technical assistance. This work was funded by the French Agence Nationale de la Recherche (ANR-11-CESA-000) and Le Fonds européen de développement régional (FEDER).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2015.12.003.

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