Short and long term neurotoxic effects following lactational exposure to the sum of the six non-dioxin-like polychlorinated biphenyls (∑6 NDL-PCBs) at low levels in offspring mice

Dissertation for the degree of Doctor of Science, Faculty of Science and Technology III, chemical, pharmaceutical, biotechnology and materials science from the University of Saarland

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Saarbrücken

Day of the conference: 23 october 2012

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Abstract

In this study, the neurotoxicity of lactational exposure to a representative mixture of the six indicator non-dioxin-like-polychlorinated biphenyls ($\sum$6 NDL-PCBs) found in contaminated fish matrices was assessed in neonatal to adult mice. We compared the performance of mice lactationally exposed to $\sum$6 NDL-PCBs (1, 10 or 100 ng/kg) to that of mice lactationally exposed to vehicle, in several developmental, behavioral and cognitive tests. Our results showed that some alterations were sex dependant; neonatal offspring female mice exhibited significantly longer turning reflexes while male mice showed a reduction in their general activity compared to the controls. Nevertheless, the exposed young and adult mice exhibited persistent anxious behavior that was detected at more progressive life stages. This persistent anxious behavior could be related to the overexpression of RyR_3 in the cerebellum *via* the disruption of calcium signaling in the neurons. Early exposure to $\sum$6 NDL-PCBs has also induced oxidative stress and cell death in juvenile mice cerebral neural and glial cells. Transcriptomic analyses have shown that the expression of proteins involved in exocytosis, social and reproductive behaviors (syt10 and vmn2r123), were significantly increased in cerebellar neural $\sum$6 NDL-PCB-exposed cells compared to the controls. Therefore, our results suggest that regular consumption of contaminated fish matrices by lactating women could be detrimental to the neurodevelopment of their newborns.

Acknowledgments

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I. Introduction

In tandem with rapid industrial and economic development, human activities have instigated widespread pollution in the natural global environment through the large distribution of anthropogenic organic pollutants. Exposure to these chemical substances may result in such adverse effects as to harm livings and hazards to human health. The occurrence of the so-called persistent organic pollutants (POPs) in the environment has raised a great concern in the last decade. Indeed, 2/3 of the food risks in industrialized countries are allotted to the animal food matrices contaminated by POPs. Some of these chemicals remain stockpiled, are produced or used illegally and deposited far from their place of issue due to their high lipophilicity and long-range transport capacities (Kang et *al*., 2009). Because of their lengthy half-lives, their bioaccumulation in fatty tissues and magnification across food chains (Fernandez and Grimalt, 2003), they continue to exist in all environmental media and biota (Cariou et *al*., 2008; Parrish et *al*., 2009). Concern centers around POP impacts on top predator species including humans. The Stockholm Convention, which acts for the stop of the production and use of harmful chemicals, had listed twelve compounds obtained by chemical synthesis, including eight kinds of pesticides (e.g. dieldrin, endrin), two kinds of industrial chemicals [e.g. polychlorinated biphenyls (PCBs), hexachlorobenzene] and two kinds of byproducts (e.g. polychlorinated dibenzo-p-dioxins) (UNEP, 2001). In May 2009, nine new POPs have been added to the original Stockholm Convention, and include four organochlorine compounds (e.g. α-, β-, γ-hexachlorocyclohexane, chlordecone), endosulfan, hexabromodiphenyl, octabromodiphenyl ether, pentachlorobenzene, pentabromodiphenyl ether, perfluorooctane sulfonate, and short-chained chlorinated paraffins (SCCPs) (UNEP, 2009).

Currently, evidence is provided that early exposure to these chemicals may increase the risk of toxic effects in life. The toxic effects of pollution are unfortunately real, as different studies provided the link between the concentrations of xenobiotics found in cord blood or in

breast milk and the induction of subsequent pathologies on human health (Crinnion, 2009). Newborn babies are particularly exposed to these toxins during their development. In addition, during this period, the central nervous system is still immature and particularly more susceptible to environmental insults than of an adult (Weiss and Landrigan, 2000). The protective barrier present in adult brains and preventing the entry of chemicals from the bloodstream into brain tissue is immature in the fetus. Accordingly, the most intensive time of brain growth corresponds also to the most vulnerable period of the open access of pollutants into brain cells (Rice and Barone, 2000). Consequently, the rise of mental disorders among children and contribution of environmental toxins became a big matter of concern. The increase in childhood pathologies such as hyperactivity, memory alterations and the occurrence of Alzheimer's disease in the elderly can find one of its explanations in the early chronic exposure of pollutants and their related impacts in early stages of life (Landrigan et *al*., 2005). Such chemical early exposure has been also implicated in various impairments, including mental retardation (Schroeder, 2000), cerebral paralysis, epilepsy, and deficits in learning, memory and attention (Porterfield, 2000), attention-deficit/hyperactivity disorder (Atladottir et *al*., 2007), cognition (Calderón-Garcidueñas et *al*., 2008) and autism (Roberts et *al*., 2007). According to Grandjean and Landrigan (2006), only few industrial chemicals such as lead, methylmercury, dioxin-like polychlorinated biphenyls (DL-PCBs), are recognized as causes of neurodevelopmental disturbances whereas 200 other pollutants are known to induce clinical neurotoxic effects in adults.

II. *State of the Art*

Among POPs, over 1.2 million tons of PCBs have been produced worldwide, since 1929, under international brand names such as Aroclor in USA and UK, Clophen in Germany or Pyralène in France (WHO, 1993). Despite being banned in 1977, they are still leaked into the environment from old electrical equipments (ATSDR, 2000) and still being produced in

numerous Asian, African and European countries (UNEP, 2003). PCBs have been produced worldwide due to their chemical stability and capacity to store electrostatic energy. They have been used in several applications (e.g. heat-exchange fluids in electrical transformers and capacitors, pesticide extenders) (UNEP, 2003), but they are also obtained as by-products during the combustion of materials containing chlorine in any form and during the manufacture of various chlorine-containing chemicals, such as ethylene dichloride. In the atmosphere, the reaction with hydroxyl radicals is the most dominant transformation process, providing PCBs half-lives from 10 days to 1.5 years, depending on the congener (WHO, 2003). These compounds are still a major environmental concern because of their ubiquity, their long-range transport, their bioaccumulation (Batterman and Chernyak, 2009; Kang et *al*., 2009) and their toxicity (Berger and Lombardo, 2001; Newman et *al*., 2009; Radice et *al*., 2008; Walkowiak, 2001). Human exposure to PCBs is mainly due to food ingestion, but it can also be attributed to inhalation and dermal contact. In mammals, PCBs are extensively absorbed from the gastrointestinal tract and primarily distributed to liver, lipid fractions and peripheral blood (ATSDR, 2000) and to a lesser extent to extrahepatic tissues (Bergman et *al*., 1982; Tanabe et *al*., 1981), undergoing further phase I metabolism conjugated to phase II metabolism (Routti et *al*., 2008). They are stored in adipose tissues and excreted *via* urine and feces. PCBs which are not metabolized or excreted are stored in fatty tissues, including brain (Dewailly et *al*., 1999). For example, Hydroxyl-PCBs (OH-PCBs) may cross the blood-brain barrier during the perinatal stage in particular (Kimura-Kuroda et *al*., 2007; Kunisue et *al*., 2007; Montie et *al*., 2009).

PCBs comprise 209 congeners which are divided into 2 subgroups: 12 coplanar compounds, called dioxin-like PCBs (DL-PCBs: PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189) and 197 non coplanar compounds or non-dioxin-like PCBs (NDL-PCBs) of which PCBs 28, 52, 101, 138, 153 and 180 are the most widespread congeners due to their significant occurrence in environmental matrices and animal tissues (EFSA, 2005,

2010; Stenberg and Andersson, 2008). It has been shown that PCBs represent the highly daily intake among POPs, reaching 615 ng/day per person in Sweden (Darnerud et *al*., 2006). The French national agency for food safety (Afssa) has fixed the tolerable daily intakes (TDI) for all 209 PCB congeners at 20 ng/kg (Afssa, 2003). This value was based upon the toxic effects induced by commercial mixtures such as Aroclors on animals like monkeys (Rice and Hayward 1997, 1999; Arnold et *al*., 1993a,b, 1995; Tryphonas et *al*., 1989, 1991) and on children during lactational exposure (Tilson et *al*., 1990). Taking into account that the 6 NDL-PCB indicators mentioned above represent about 50 % of the total PCBs, Afssa has set a TDI at 10 ng/kg (Afssa, 2007). Concerning DL-PCBs, maximum levels have been fixed at 2.33 pg toxic equivalent quantity (TEQ)/kg body weight/day but there are no restrictions concerning NDL-PCBs (Afssa, 2007, 2008; VKM, 2008), although, maximum levels in the ∑6 NDL-PCBs were proposed in some foodstuffs, including fish (125 ng/g fresh weight) (UE settlement No. 1259/2011). Indeed, PCB contamination is mainly due to fish consumption (57 %) (Bilau et *al*., 2007; Darnerud et *al*., 2006). Contaminated fish contains 9 % of DL-PCBs and 91 % of NDL-PCBs, in which ∑6 NDL-PCBs are detected at 56 % (Bhavsar et *al*., 2007; VKM, 2008). PCB 153 and PCB 138 are the predominant congeners in the ∑6 NDL-PCBs representing ca. 37 % and 32 % of the total compounds, followed by PCB 101 (12 %), PCB 180 (11 %), PCB 52 (6 %) and PCB 28 (2 %) (Karl et *al*., 2010; Naso et *al*., 2005; Santillo et *al*., 2005; Szlinder-Richert et *al*., 2009; Van der Oost et *al*., 1996).

The NDL-PCBs, which impair the AhR binding, is thought to induce different toxicological properties than DL-PCBs. Thus, NDL-PCB modes of actions are mostly still undiscovered in particular on the central nervous system. According to Kodavanti (2005), NDL-PCB congeners and/or their metabolites could induce neurotoxic effects (developmental and behavioral impairments), through the alteration in thyroid hormone homeostasis, disruption in intracellular signaling, disturbance in neurotransmitter levels and changes in gene expression. Indeed, it has been shown that individual NDL-PCBs at high levels are able

to induce neurological and behavioral alterations (Boix et *al.*, 2010, 2011; Colciago et *al.*, 2009; Eriksson and Fredriksson, 1996; Haave et *al.*, 2011). These pollutants alter neurotransmitter receptor levels such as dopamine, gabaergic, ryanodine, and serotonin receptors (Fernandes et *al.*, 2010; Pessah et *al.*, 2010), which may possibly underlie neurobehavioral effects observed *in vivo,* such as changes in motor activity, learning, memory, depression, anxiety and cognition (Boix et *al.*, 2010, 2011; Ide et *al.*, 2010; Kouzu et *al.*, 2000; Ogren et *al.*, 2008).

Interestingly, NDL-PCBs are detected at high levels in breast milk (Cerna et *al.*, 2010; Devanathan et *al.*, 2012; Skrbic et *al.*, 2010). The lactational transfer represents the primary route of PCB exposure to developing mammals because the majority of maternal PCB 153 burden is transferred to the pups during lactation (Lee et *al.*, 2007). Consequently, it becomes increasingly relevant to study the neurotoxic effects induced by low doses of mixtures of NDL-PCBs because they exhibit unclear modes of actions on the juvenile central nervous system.

In this study, the toxicity induced by lacational exposure to $\sum 6$ NDL-PCBs in the central nervous system of mice offspring was evaluated *in vivo* by performing classical developmental, behavioral and cognitive tests. NDL-PCB-induced cytotoxicty, including oxidative stress and cell viability was assessed in peripheral and central cells of juvenile mice offspring by flow cytometric measurements. The neurobiological changes following early exposure to PCBs were analyzed in the different brain parts of the juvenile brain by assessing the gene expression of enzymes and receptors and by conducting transcriptomic analyses on isolated cerebellar cells.

III. Materials and Methods

III.1. Animals and general study design

We used sexually mature Swiss albino female and male mice (OF1, Charles River, France), aged 9 weeks (30-40 g). They were kept in standard cages at 5 mice/sex/cage, under a reversed light/dark cycle (light on from 8:00 p.m. to 8:00 a.m.), at a constant temperature ($22 \pm 2°C$) and a relative humidity (55 ± 10 %), with free access to food pellets (SDS Dietex, France) and tap water. After two weeks of acclimatization, two female mice were mated with one male overnight and were examined the following morning for copulatory plugs. We singly housed female mice on the day on which a vaginal plug was present. One week before the delivery, we supplied a cotton nest square as source of nesting material. The day of parturition was considered to be the postnatal day (PND) 0 where we recorded sex and individual pup weight. We reduced litters to ten pups, equally composed of both sexes. On PND 0, we allocated nurturing females to experimental groups by stratified randomization to obtain an equivalent mean of body weights of each litter across all groups. We treated dams daily with the $\sum 6$ NDL-PCBs using feeding needles for oral gavage into their stomachs during the lactational period (PND 0 to PND 21).

The 6 NDL-PCBs (28, 52, 101, 138, 153 and 180) were obtained from Sigma–Aldrich Co. (St. Quentin Fallavier, France, purity > 99 %). We prepared a stock solution of the $\sum 6$ NDL-PCBs according to the percentages of each of the NDL-PCB congeners in the mixture found in contaminated fish matrices (10 mg NDL-PCB mixture in 10 ml of rapeseed oil). Then we subsequently diluted (1:10) the stock solution of the $\sum 6$ NDL-PCBs in rapeseed oil to obtain the following doses: 1, 10 and 100 ng/kg (table 1). Rapeseed oil alone was used for control dams. The administration volume was 10 ml/kg body weight.

∑6 NDL-PCBs	Indicator NDL-PCBs					
Level (ng/kg)	CB 153 (ng/kg)	CB 138 (ng/kg)	CB 180 (ng/kg)	CB 101 (ng/kg)	CB 52 (ng/kg)	CB 28 (ng/kg)
1	0.37	0.32	0.11	0.12	0.06	0.02
10	3.70	3.20	1.10	1.20	0.60	0.20
100	37.00	32.00	11.00	12.00	6.00	2.00

Table 1. Selected levels and formulation of the NDL-PCB mixtures based on the occurrence of individual NDL-PCB congeners in contaminated fish. The main dose level of the NDL-PCB mixture used corresponds to low levels extrapolated from human data (TDI of 10 ng/kg corresponding to 100 ng/kg in mice) with two lower levels (1 and 10 ng/kg in mice).

We conducted the weighing, neuromotor maturation and post-weaning tests in one pup of both sex randomly selected from each litter (n = 20/group) according to OECD guidelines for the testing of chemicals. The overview of the general study design is given in figure 1. For the gene expression and oxidative status analysis, one pup per litter per sex was picked randomly, euthanized with halothane and decapitated on PND 14 (end of exclusive breastfeeding period) or on PND 21 (weaning). Blood was collected in tubes and regions of interest were excised from the fresh brain. Some samples were directly stored at -80°C for the quantification of gene expression analysis. For the cytometric measurements, mice tissues were freshly used. After weaning, mice were housed with the same-sex littermates in groups of five mice per cage (OECD, 2007). All animal procedures were carried out in accordance with the European Union (Directive 2010/63/EU).
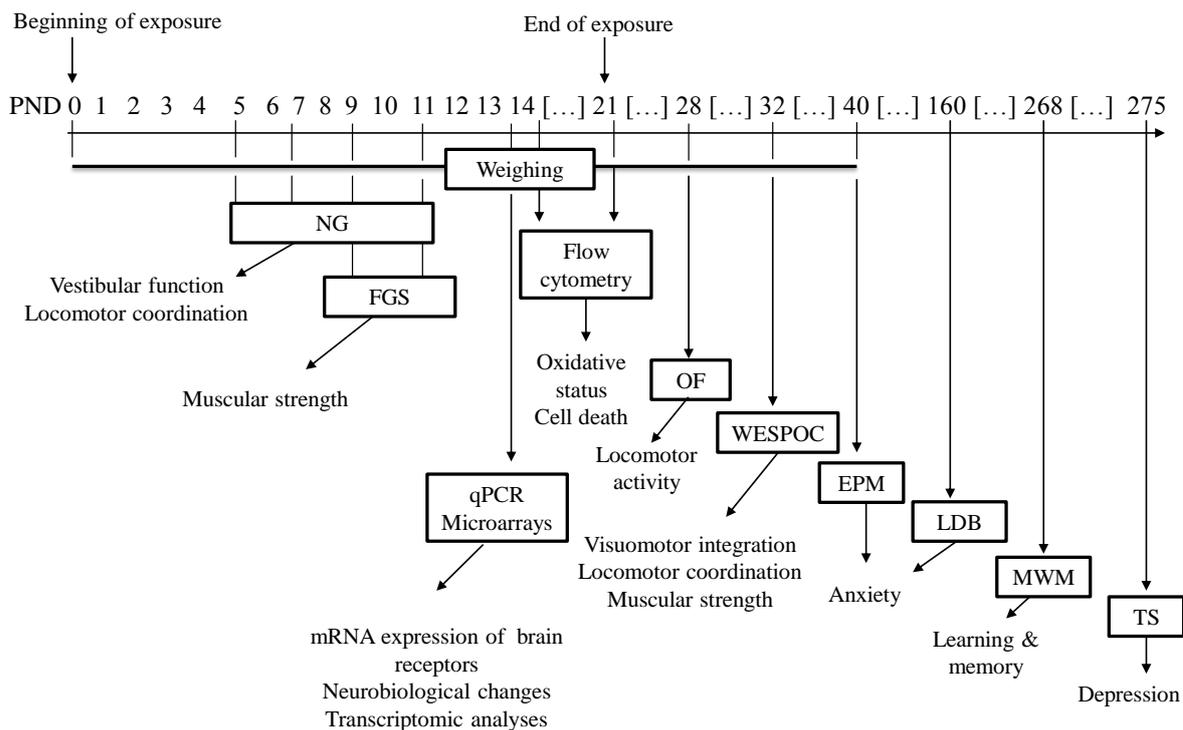
Figure 1. Schematic diagram of the experimental design performed on ∑6 NDL-PCB lactationally exposed mice and controls. The experimental design considered the age of the offspring mice and aimed to measure early effects by conducting developmental tests in pups (negative geotaxis (NG) and forelimb grip strength (FGS) tests) as well as late effects using behavioral and cognitive tests in young (water escape pole climbing (WESPOC) and open field (OF) tests) and adult offspring mice (elevated plus maze (EPM), light-dark box (LDB), Morris water maze (MWM), tail suspension (TS) tests). The EPM and LDB tests were performed separately at distinct developmental periods (postnatal days (PNDs) 40 and 160) to evaluate whether the anxious behavior was persistent over time. Quantitative real-time polymerase chain reaction (qPCR) and microarrays were performed at the end of the exclusive breast-feeding period in mice (PND 14). The oxidative status and the cell viability on central and peripheral cells were evaluated in mice on PNDs 14 and 21 by flow cytometry technique.

III.2. Functional study

III.2.1. Body weight

The dams were weighed daily during nursing in order to adjust the levels of exposure and to report any sign of PCB toxicity through maternal weight loss. The offspring body weight was measured on PNDs 0, 4, 8, 12, 16, 20, 26 and 40 for the physical development assessment.

III.2.2. Maternal behavior

As the exposure to ∑6 NDL-PCBs during lactation may have deleterious consequences on maternal behavior that could influence pups' development, we conducted a nest building activity and retrieving test during this period as described previously (Bouayed et *al*., 2009a; Elnar et *al*., 2012). Briefly, the quality of the nest was assessed every 2 days from parturition using a 3-point scale (0 = no nest or primitive flat nest; 1 = complex cup-shaped nest; 2 = complex hooded nest). The second test consists in placing the pup alone at a 20 cm distance from the nest. We recorded the latency time during which the mother retrieved the pup and carried it back to the nest. The mean time of two trials was used for analysis.

III.2.3. Developmental, behavioral and cognitive tests

III.2.3.1. Negative geotaxis (NG) test

This test conducted on PNDs 5, 7, 9 and 11 aimed to assess both motor coordination and vestibular function (Pryor, 1983). Each animal was given one trial (120 s) in which we measured the time taken by the pup to complete a 180-degree turn when it is placed in a head-down position on a 15-degree inclined plywood surface.

III.2.3.2. Forelimb grip strength (FGS) test

This test was used to evaluate the muscle strength (Nevins et *al*., 1993). A metal rod (0.5 mm in diameter) was stretched between the two poles of a frame at about 20 cm above the table. The pup was suspended by its front paws holding the metal rod in a container with cotton to prevent it from falling once it released the rod. The maximum time the animal remained hanging from the rod on two consecutive trials was recorded, without any time limit. This test was carried out on PNDs 9 and 11, before eyelid opening, to control musclar strength and to eliminate the possible influence of emotivity.

III.2.3.3. Open field (OF) test

We used a circular open field platform, with the floor divided into 36 squares delimiting peripheral, medium and central circular areas, to assess general locomotor activity in young mice on PND 28 (Hall and Ballachey, 1932). We placed the mice with their heads in front of the apparatus wall and after 1 min of habituation we recorded the number of squares crossed with the four paws (horizontal activity) and the number of rearings (vertical activity) for 3 min.

III.2.3.4. Water escape pole grasping (WESPOC) test

This test was conducted on PND 32 in order to assess the visuomotor integration (Lister, 1990). The apparatus of the WESPOC test is composed by a basin with a rigid rope placed in its center and a circular platform 20 cm above water level with a circular opening 8 cm in diameter. Mice were placed with their heads facing the wall of the basin. The times spent to grasp the rope, to climb upward, to escape the pole and to reach a normal quadruped position on the platform without falling were recorded (Essman and Jarvik, 1961). This 3-step test requires considerable musclar strength and coordination of all four limbs. In each step, mice were given 180 s to accomplish the task.

III.2.3.5. Elevated plus maze (EPM) and light-dark box (LDB) tests

The level of anxiety in mice was assessed on PND 40 using the EPM test (Lister, 1990). The mouse was placed in the central square of the apparatus, facing one of the closed arms and was allowed to walk freely. We measured the following behaviors for 5 min: the number of attempts to enter an open arm, the latency time of the first entry into an open arm, the total number of entries and the cumulative time spent in the open arms and the total number of visits to the closed arms. The proportions of the time spent in the open arms and the entries into open arms were expressed in percentages.

The LDB test is based on an innate aversion of rodents to novel, brightly illuminated spaces and is used to assess anxiety-related behaviors in rodents (Bourin and Hascoet, 2003). It was conducted on PND 160 to evaluate the persistence of the effects observed in the EPM test. Briefly, we placed the mice in the lit box, head towards the dark box, allowing them to explore the new apparatus for 3 min. We recorded the latency to emerge from the dark box, the number of attempts in the lit box, the number of entries and the time spent into the lit box, the number of squares crossed and the total time spent in the dark box.

III.2.3.6. Morris water maze (MWM) test

We conducted this test on PND 268 in order to assess the late effect of an early lactational exposure to $\sum 6$ NDL-PCBs on learning and memory retention in adult mice. The apparatus is a large circular pool of water (t = 24°C), divided into four quadrants (Morris, 1984) where we placed four visual clues and a hidden platform, just below (1.0 cm) the surface of the water mixed with milk, in the center of one of the quadrants. We recorded the escape latency to reach the hidden platform for five trials. After reaching the platform, the mouse was left for 30 s. We interrupted the trial if a mouse did not reach the platform within 120 s, defined the escape latency as 120 s and guided the mouse to the platform. We evaluated memory retention by performing one trial after 24 h.

III.2.3.7. Tail suspension (TS) test

The TS test which was conducted on PND 275 is one of the most common animal models of depression (Cryan et *al*., 2005). Briefly, we suspended mice by their tails with a tape and we recorded the duration of their behavioral immobility for 6 min. They were positioned such that the base of their tail was perpendicular to the bar.

III.3. Cellular mechanism: cell differentiation and flow cytometric measurements

Male pups were randomly selected from each litter, anaesthetized with halothane and then sacrificed (PNDs 14 and 21). On PND 14, only cerebellum of male mice was excised from the brain in order to isolate four distinct cells: astrocytes, oligodendrocytes, neural cells and microglia according to the manufacturers' protocol (Miltenyi, Biotec). These cells were used for oxidative status evaluation and for further transcriptomic analyses.

On PND 21, blood and three brain regions were collected in tubes. The preparation of blood has been carried out as described by Bouayed et *al*., (2007) and Rammal et *al*., (2008a). The regions of interest (cortex, cerebellum or hippocampus) were excised from the fresh brain, gently disrupted and homogenized in 1 x PBS. The following protocol was performed according to Rammal et *al*., (2008b) which was based on the manufacturer's recommendations (Miltenyi Biotec, France). After tissue disruption and homogenization, an appropriate MINIMACS MS column was used for the magnetic separation of cells. For example, neural stem cells express Prominin 1; they are consequently magnetically separated from other cell types in the mouse brain using anti-Prominin-1 microbeads in the MACS® Column Separator (Miltenyi Biotec, France) and magnetically labelled prominin-1^{+} cells are retained on the column. The same principle was applied to astrocytes (Anti-GLAST), oligodendrocytes (Anti-O4), microglia (CD 11b) and neuronal cells (CD 90.2). At the end of the protocol, 80 µl of the isolated cells were mixed with 5 µl of DCFH-DA (50 µM) and incubated at 37°C for 15 min, while 20 µl were suspended in 5 µl Annexin V and 5 µl of propidium iodide (PI) and were incubated away from light at room temperature for 15 min in order to evaluate the induction of apoptosis and necrosis.

We used a flow cytometer (FACScan, Becton-Dickinson, Immunofluorometry Systems, France) to distinguish cells according to their size (forward light scatter, FSC) and relative granularity (side light scatter, SSC) after their excitation with a 488 nm argon laser beam. We took into account the emitted mean fluorescence intensity (MFI) in channel 1 (FL1: 525 nm)

detection (8000 to 20000 events) as a measurement of the levels of intracellular reactive oxygen species using DCFH-DA. Discrimination among viable, apoptotic and necrotic cells were performed *via* FL1 (525 nm) and FL2 (575 nm) detection on 8000 events.

III.4. Molecular mechanism

III.4.1. mRNA expression of 5-HT$_{1A}$, GABA$_{A\alpha1}$, μ-OR$_1$ and RyR$_3$

We performed total RNA isolation from three brain regions (cortex, cerebellum and hippocampus), reverse transcription and EvaGreen quantitative real-time polymerase chain reaction (qPCR) as detailed by Tybl et *al*., (2011) and Elnar et *al*., (2012) and according to the manufacturers' instructions (Qiazol Lysis Reagent, Qiagen, Hilden, Germany; DNA free Kit, Ambion, Germany; High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Darmstadt, Germany, respectively). Cyclophilin (Cyc), 18S, 5-hydroxytryptamine serotonin 1A (5-HT$_{1A}$), μ 1-opioïd (μ-OR$_1$), ryanodine 3 (RyR$_3$), and gamma-aminobutyric acid A A alpha1 (GABA$_{A\alpha1}$) cDNA sequences were acquired and designed from NCBI GenBank and NCBI Primer BLAST. We performed a conventional PCR including a positive control to check for residual contamination of isolated RNA with genomic DNA. Samples prepared without reverse transcriptase served as negative controls in the subsequent qPCR.

Real-time PCR were run in triplicates, using 4 µl 5 x HOT FIREPol[®] EvaGreen[®] qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) in an iQ5 cycler (Bio-Rad, Munich, Germany). The reaction volume was 20 µl including 4 µl of the kit, 0.2 µl - 0.4 µl of each of the forward and reverse primers and 4 µl cDNA. Primer sequences and annealing temperatures are included in supplementary table 1. The amplification conditions for 40-45 cycles were as follows: 95°C for 30 min, 95°C for 20 s, 58-63°C for 20 s and 72°C for 20s. We carried out melt curve analyses from 55 to 98°C for each sample and gene to distinguish specific from non-specific products. The quantification cycle (Cq) was determined by the instrument software (Bio-Rad). It represents the PCR cycle at which an increase in reporter fluorescence above background is

first detected. We included a (1:5) cDNA standard curve in each run to calculate the gene-specific PCR efficiency and the relative gene expression. Details about qPCR validation are given in supplementary table 1. We tested two reference genes (Cyc and 18S) for normalization purposes with geNORM software. The relative expression of each gene of interest was normalized to the relative gene expression of 18S because it was not affected following the lactational exposure to $\sum 6$ NDL-PCBs.

III.4.2. mRNA expression of Cat, CuZnSOD$_1$, Glo$_1$, GPx$_1$ and Gsr$_1$

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 (following the manufacturer's instructions). Quality control was performed with the RNA Nano 6000 assay (Agilent Technologies, Diegem, Belgium) using a 2100 Bioanalyzer (Agilent Technologies). RNA samples with a RNA integrity number (RIN) lower than 7 were excluded from the experiment. RNA purity and concentration were assessed measuring the absorbance at 230 nm, 260 nm and 280 nm using a Nanodrop ND-1000 spectrophotometer (Thermo scientific, Villebon-sur-Yvette, France).

We used five target genes [catalase (Cat), copper zinc superoxide dismutase 1 (CuZnSOD$_1$), glyoxalase 1 (Glo$_1$), glutathione peroxidase 1 (GPx$_1$), glutathione reductase 1 (Gsr$_1$)] and six housekeeping genes [actin b (Actb), aryl hydrocarbon receptor interacting protein (Aip), CXXC finger protein 1 (Cxxc1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mitochondrial ribosomal protein L48 (Mrpl48), RNA polymerase II (RpolyII)]. Primer sequences that were selected from Primer Blast Genbank and used in this study are listed in supplementary table 1.

Reverse transcription was performed using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, NM, USA) from 1 µg RNA following the manufacturer guidelines in a 20 µl final volume. PCR was performed using Mesa Green Low Rox Real-time PCR Kits

(Eurogentec, Liège, Belgium) with the following final concentrations in 25 µl final volume: 1x Master Mix, 100 nM forward and reverse primers, 0.4 ng.µl^{-1} cDNA on a 7500 Fast system (Applied Biosystems). Thermal cycling conditions were: initial 5 min denaturation at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C, and a final dissociation step. Primer specificity was controlled by the presence of a single peak in the melting curve and PCR efficiency was assessed using decreasing five-fold dilution (from 25 ng to 0.04 ng and no cDNA).

Relative expression was calculated taking into account 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).

III.4.3. Transcriptomic analyses in isolated cells

The total RNA was extracted from cells to 25 and 100 µg/ml of isolated cells for 24 h using RNeasy Mini Kit (QIAGEN, Courtaboeuf, France) following manufacturer instructions. RNA quality and concentration were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). All experiments were performed in triplicate.

Microarray experiments were performed following the MIAME (Minimal Information About a Microarray Experiment) criteria (Brazma et *al*., 2001). Briefly, RNA samples were extracted from cells, amplified and labeled using One-Color Gene Amp Labeling Kit (Agilent Techologies, Massy, France) following manufacturer's instructions. RNA was denatured and reverse transcribed to cDNA. These cDNA were transcribed *in vitro* to RNA using T7 RNA polymerase and labeled with fluorescent cyanine 3-CTP. Fragmented cDNA was hybridized to Agilent 4 x 44 K Whole Mouse Genome Microarray (Agilent Technologies, Massy, France). Microarrays performed in triplicate were washed, stabilized and dried using the acetonitrile and Stabilization and Drying Solution (Agilent Technologies, Massy, France).

The slides were scanned and images were extracted using Feature Extraction Software version 9.5.3 (Agilent). Analyses were performed by GenespringGX12 (Agilent).

III.5. Statistical analyses

The distribution was checked for each variable using Kurtosis and Skewness test. The data with a Gaussian distribution were analyzed using parametric analysis of variance (ANOVA). *Post hoc* analyses were performed using the Fisher's test when one-way ANOVAs were significant ($p < 0.05$). Data are reported as mean ($\pm$ SEM) and significance was set at $p < 0.05$.

When data did not follow a Gaussian distribution, non-parametric statistics using Kruskal-Wallis test, were conducted. *Post hoc* analyses between controls and $\sum 6$ NDL-PCB-exposed groups were performed following the Mann-Whitney *U*-test except for mRNA expression analysis, where a Mann-Whitney test for multiple comparisons was performed (Siegel and Castellan, 1988). Data are reported as median (IQR). Significance was set at $p < 0.05$.

All statistical analyses were carried out using the Statview® 4.5 statistical package (Abacus Concepts, Inc.).

Microarray data and statistics were analyzed using GeneSpringGX12G software (Agilent Technologies, Palo Alto, CA, USA). Regarding qRT-PCR and array results, genes with over a 2- fold or less than 0.5- fold intensity ratio, compared with those of unexposed control, were seen as up- or down-regulated, respectively. The differences between the control and the experimental group were evaluated with the one-paired *t*-test. *P*-values less than 0.1 were considered as significant. Gene Ontology (GO) extraction was performed with the same significance ($p < 0.1$).

IV. Results

IV.1. Effects on body weight

There were no signs of toxicity in treated dams during the whole experimental period or any significant effect on body weight gain between the ∑6 NDL-PCB-exposed dams and the controls [treatment: $F(3,216) = 1.64$, $p = 0.18$; treatment × time: $F(15,216) = 0.78$, $p = 0.68$] (supp. table 2). Nevertheless, a significant effect of time was observed indicating that dams gained weight over time [$F(5,216) = 59.34$, $p < 0.0001$].

No mortality was noticed among pups following lactational exposure to ∑6 NDL-PCBs. We analyzed male and female pups separately because weight gain in mice is sex-dependant. We noticed that exposure to ∑6 NDL-PCBs during nursing induced a significant body weight gain only in males at 100 ng/kg [PND 4: $z = -2.04$, $p = 0.04$; PND 8: $z = -3.25$, $p = 0.001$; PND 12: $z = -3.78$, $p = 0.0002$] and females at 10 ng/kg [PND 4: $z = -2.12$, $p = 0.03$; PND 8: $z = -2.27$, $p = 0.02$; PND 12: $z = -2.34$, $p = 0.02$; PND 16: $z = -2.57$; $p = 0.01$; PND 20: $z = -2.42$, $p = 0.02$] in comparison to the control pups. These differences were no more observed after weaning (PND 26 –PND 40) (fig. 2).
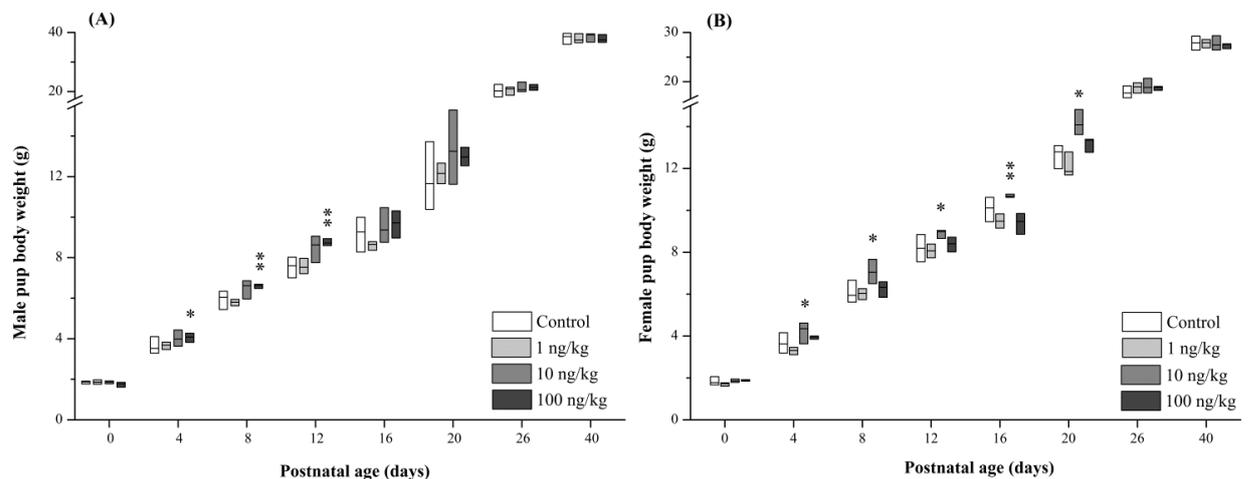
Figure 2. Postnatal evolution of the body weight [g] of male (A) and female (B) mice that were lactationally exposed to 1, 10 and 100 ng/kg ∑6 NDL-PCBs compared to controls (n = 10/sex/group). *p < 0.05, **p < 0.01. The data are reported as the median (IQR: 25-75).

IV.2. Effects on maternal behavior

The exposure to $\sum 6$ NDL-PCBs did not induce any significant effect on the nest building activity in dams when compared to the controls [$F(3,108) = 1.06$, $p = 0.36$] while a significant decrease was found across all of the groups in the nest quality at the end of the lactational period indicating the onset of weaning [time: $F(5,108) = 11.85$, $p < 0.0001$]. There was no interaction between time and treatment [$F(15,108) = 1.45$, $p = 0.20$]. No significant effect was observed on the retrieving time among all groups during the nursing [treatment: $F(3,72) = 0.45$, $p = 0.71$; treatment $\times$ time: $F(3,72) = 0.52$, $p = 0.60$] (supp. table 2).

IV.3. Effects on vestibular function, locomotor coordination and muscular strength

We observed significant effects for treatment and age [$F(3,288) = 4.86$, $p = 0.0026$; $F(3,288) = 5.60$, $p = 0.0006$, respectively] but not for sex [$F(1,288) = 0.64$, $p = 0.42$] in the performances of the pups in the NG test. However, the interactions between the three variables revealed that the effects of lactational exposure to $\sum 6$ NDL-PCBs depended on sex [treatment $\times$ sex: $F(3,288) = 3.35$, $p = 0.01$; treatment $\times$ sex $\times$ time: $F(9,288) = 1.93$, $p = 0.04$]. However, there were no significant differences between the performances of male pups lactationally exposed to $\sum 6$ NDL-PCBs when compared to controls on any day (fig. 3A). Regarding female pups, only those whose mothers were exposed to $\sum 6$ NDL-PCBs at 100 ng/kg required significantly more time to turn completely up in comparison to the controls. These differences were transient because they were observed starting on PND 7 ($p = 0.001$) and PND 9 ($p = 0.002$), but they disappeared on PND 11 ($p = 0.17$) (fig. 3B).

In the FGS test, ANOVA revealed that there were no significant differences in the performances of lactationally $\sum 6$ NDL-PCB-exposed pups in comparison to the control pups across all the groups [$F(3,142) = 0.39$, $p = 0.75$], indicating that the treatment did not affect their muscular strength. In addition, the performances of the pups did not depend on the sex [$F(1,142) = 0.54$, $p = 0.46$] but on the age of the pups [$F(1,142) = 9.35$, $p = 0.0027$].

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Nevertheless, no interactions between the main studied factors were found [treatment $\times$ sex: $F(3,142) = 0.70$, $p = 0.54$; treatment $\times$ time: $F(3,142) = 0.81$, $p = 0.48$; treatment $\times$ sex $\times$ time: $F(3,142) = 0.52$, $p = 0.60$] (supp. table 3).
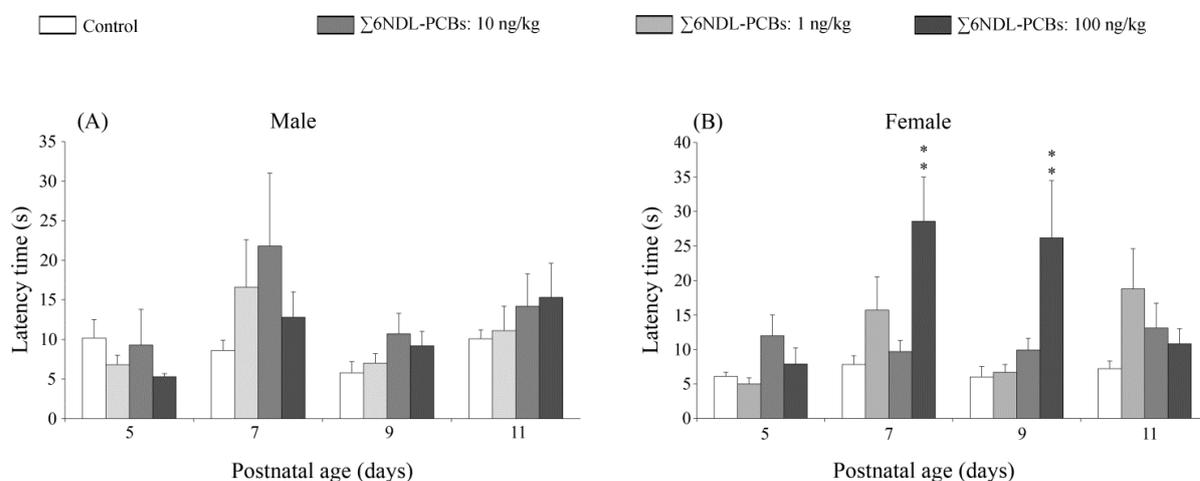
Figure 3. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls on the turning reflex of male (A) and female (B) mice (PNDs 5, 7, 9 and 11) in the negative geotaxis test (NG, n = 10/sex/group). The data are reported as the mean ± SEM. **p < 0.01 significantly different from the control.

IV.4. Effects on locomotor activity

In the OF test, the horizontal locomotor activity of young mice tends to decrease following lactational exposure to ∑6 NDL-PCBs [$F(3,72) = 2.28$, $p = 0.08$]. A significant effect was observed on sex [$F(1,72) = 6.75$, $p = 0.011$] with an interaction between sex and treatment [$F(3,72) = 4.5$, $p = 0.005$]. When animals were analyzed separately, no significant effect was found in young females [$F(3,36) = 0.17$, $p = 0.91$], while young males whose mothers were exposed to 1 and 10 ng/kg of ∑6 NDL-PCBs ($p = 0.002$ and $p = 0.001$, respectively) have shown a strong decrease in their locomotor activity [treatment: $F(3,36) = 8.93$, $p = 0.0001$] (fig. 4). However, there were no significant effects of treatment [$F(3,72) = 1.23$, $p = 0.30$], sex [$F(1,72) = 2.52$, $p = 0.12$], or interaction [$F(3,72) = 0.91$, $p = 0.44$] on the number of rearings (supp. table 3).
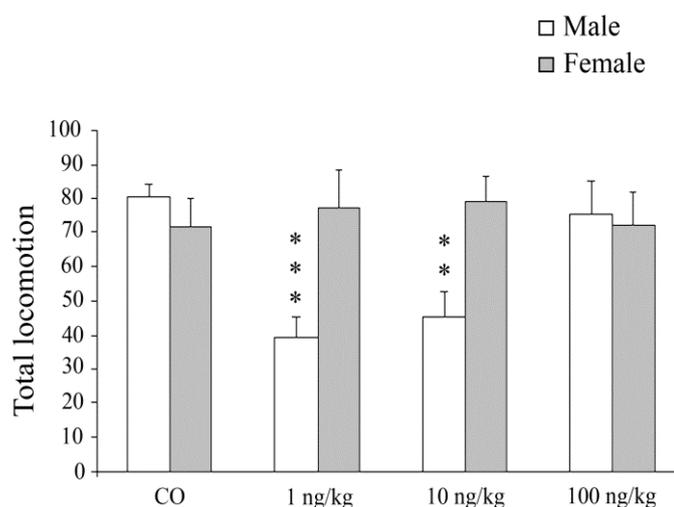
Figure 4. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls (CO) on the behavior of young mice (PND 28) in the open field test (OF, n = 10/sex/group). The data are reported as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 significantly different from the control.

IV.5. Effects on visuomotor integration

On PND 32, the WESPOC test has revealed that the lactational exposure to ∑6 NDL-PCBs has significantly extended the swimming time of pups ($H = 9.69$, $p = 0.02$) whose mothers were exposed to 1 ng/kg ($z = -2.27$, $p = 0.02$) and 100 ng/kg ($z = -2.89$, $p = 0.004$) in both sexes. The separate analyses have shown that only young male mice were affected by the treatment ($z = -2.30$, $p = 0.02$; $z = -2.04$; $p = 0.04$, respectively) as they required more time to reach the pole in comparison to the control mice (table 2). Although female mice exposed to the highest dose also needed more time to reach the pole, this difference was not significant due to the strong variability within the experimental data ($H = 3.63$, $p = 0.30$) (table 2).

An increase in the pole grasping latency in the WESPOC test is an indication of altered visuomotor integration and/or locomotor coordination (the quality of swimming). In addition, both the exposed and control animals were blindly assessed during their swimming period by a qualified observer. No differences in swimming quality were detected, indicating that any alterations caused by the treatment were due to a lack of visuomotor integration. However, no significant effects were observed in lactationally exposed mice with regard to the climbing

time (H = 4.09, p = 0.25) and pole escape latency (H = 1.72, p = 0.63) in comparison to the

controls (table 2).

Sex	Variables	Control	$\sum$6 NDL-PCBs 1 ng/kg	$\sum$6 NDL-PCBs 10 ng/kg	$\sum$6 NDL-PCBs 100 ng/kg
Male	Pole grasping latency (s)	8.00 (7.20 – 9.58)	11.40 * (9.90 – 23.80)	8.60 (7.20 – 24.10)	22.95 * (10.70 – 65.90)
	Climbing time (s)	5.50 (4.30 – 5.90)	4.30 (2.40 – 6.70)	3.55 (2.70 – 5.80)	2.60 (2.45 – 4.25)
	Pole escaping latency (s)	4.20 (1.30 – 8.00)	3.00 (1.30 – 6.10)	3.65 (2.70 – 6.50)	5.80 (3.02 – 15.02)
Female	Pole grasping latency (s)	9.10 (7.50-11.10)	11.10 (7.60 – 20.90)	12.85 (6.70 – 37.40)	23.65 (9.40 – 67.30)
	Climbing time (s)	3.25 (2.20 – 4.70)	2.65 (2.40 – 3.70)	3.70 (3.30 – 3.80)	3.10 (2.90 – 4.00)
	Pole escaping latency (s)	14.35 (9.60 – 17.00)	16.20 (3.90 – 23.30)	10.90 (4.07 – 13.12)	10.95 (4.50 – 23.10)

Table 2. The effects of lactational exposure to $\sum$6 NDL-PCBs compared to controls on the behavior of young male and female mice (PND 32) in the water escape pole climbing test (WESPOC n = 10/sex/group). The data represent the medians and quartiles (25-75) for the different parameters recorded. Statistically significant differences between the exposed and control mice: *p < 0.05.

IV.6. Effects on anxiety

In the EPM test, the lactational exposure to $\sum$6 NDL-PCBs induced a significant effect on the latency of the first entry into an open arm [$F(3,72)$ = 3.85, p = 0.013] but there was no sex interaction [$F(3,72)$ = 1.76, p = 0.16]. The young mice whose mothers were daily exposed to 10 and 100 ng/kg $\sum$6 NDL-PCBs took significantly more time to perform the first entry into an open arm than controls (p = 0.004 and p = 0.005, respectively) (fig. 5A). Subsequent analyses revealed a strong increase in the number of attempts to enter an open arm [$F(3,72)$ = 6.13, p = 0.0009] in mice lactationally exposed to $\sum$6 NDL-PCBs, again at 10 and 100 ng/kg (p = 0.0002; p = 0.005, respectively) (fig. 5B). In addition, the treatment induced a significant effect [$F(3,72)$ = 3.50, p = 0.019] on the proportion of time spent in the open arms (%). Mice whose mothers were treated at all levels of $\sum$6 NDL-PCBs spent significantly less time in the open arms than control pups (p < 0.05) (fig. 5C). Regarding the proportion of entries into the open arms, a two way-ANOVA did not reveal any significant differences between the groups [treatment: $F(3,72)$ = 1.77, p = 0.16; treatment $\times$ sex: $F(3,72)$ = 1.20, p = 0.31] (fig. 5D).

Furthermore, there was no effect on the number of closed arm entries when the animals were analyzed together or when they were analyzed based on sex, indicating that there was no significant treatment effect on the general locomotor activity of the animals in this test (fig. 5E).
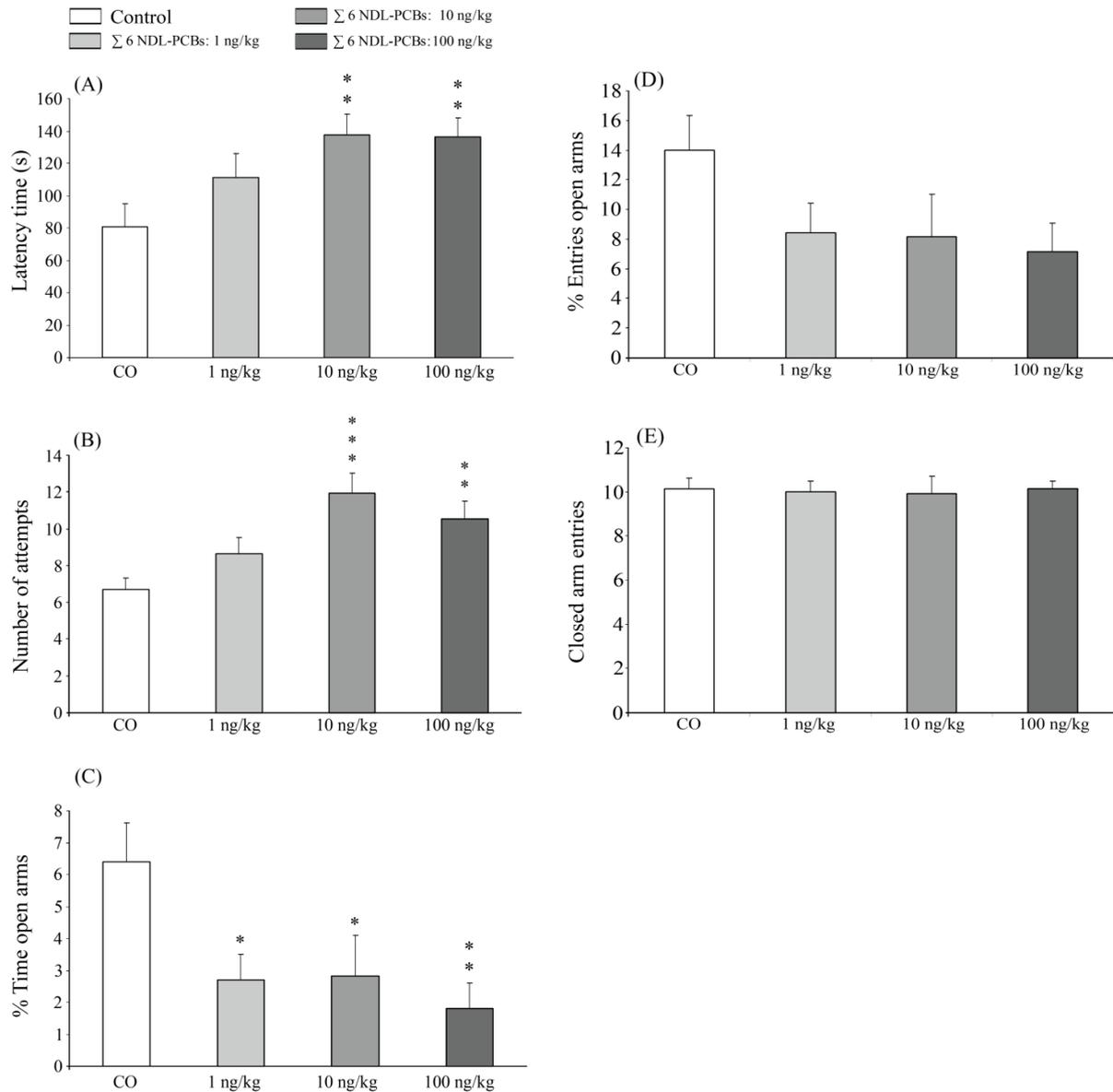
Figure 5. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls (CO) on the behavior of young Swiss mice (PND 40) in the elevated plus maze test (EPM, n = 10/sex/group). The latency of the first entry (A), the number of attempts (B), time spent in open arms (%) (C), the number of entries into open arms (%) (D) and the number of closed arm entries (E) is presented. The data are reported as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 significantly different from the control.

In the LDB test, we found significant effects of treatment on the latency time [$F(3,67) =$ 6.96, $p = 0.0004$] and on the time spent in the lit box [$F(3,67) = 4.30$, $p = 0.007$]. The $\sum6$ NDL-PCB treatment did not depend on sex [treatment × sex: $F(3,67) = 1.42$, $p = 0.24$]. Mice whose mothers were lactationally exposed to $\sum6$ NDL-PCBs (1, 10 and 100 ng/kg) took significantly more time to leave the dark box ($p < 0.0001$, $p = 0.012$, $p < 0.01$, respectively) (fig. 6A) and spent significantly less time in the lit box ($p < 0.001$, $p = 0.011$, $p = 0.03$, respectively) (fig. 6B) than controls. In addition, more transitions were made by $\sum6$ NDL-PCB-exposed mice (1 and 10 ng/kg) than the control mice ($z = -3.17$, $p = 0.0015$; $z = -2.00$; $p = 0.046$, respectively) (fig. 6C). Moreover, there was no significant effect of lactational exposure to $\sum6$ NDL-PCBs on the general locomotor activity of the animals in this test (fig. 6D) as no effect of exposure to $\sum6$ NDL-PCBs on locomotion in the dark box was revealed [treatment: $F(3,67) = 0.77$, $p = 0.51$; treatment × sex: $F(3,67) = 0.72$, $p = 0.54$].
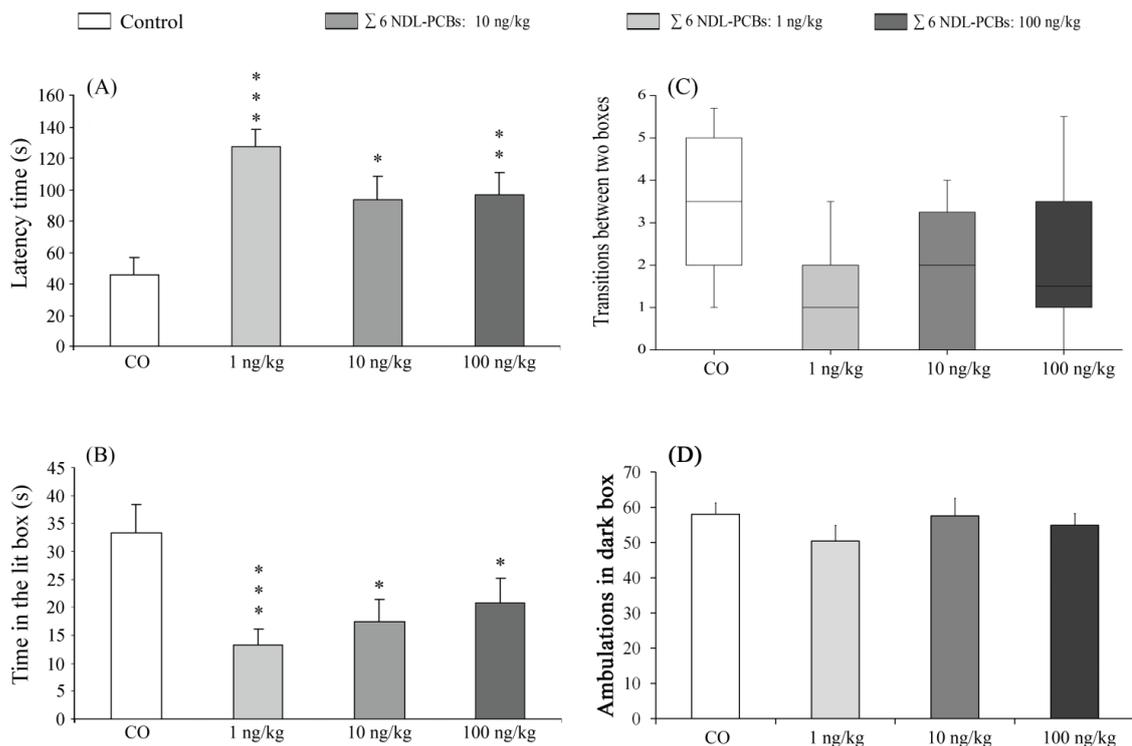
Figure 6. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls (CO) on the behavior of adult mice (PND 160) in the light-dark box test (LDB, n = 10/sex/group). The latency of the first entry (A) and the time spent in the lit box (B), the number of exits into the lit box (C) and the ambulation in dark box (D) are presented. Except for the number of exits (reported as medians and quartiles), the data are reported as the mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 significantly different from the control.

IV.7. Effects on memory and mood

The learning ability in adult mice was not affected following lactational exposure to $\sum6$ NDL-PCBs as shown in the MWM test [treatment: $F(3,320) = 0.04$, $p = 0.98$; treatment $\times$ sex: $F(3,320) = 0.11$, $p = 0.90$; treatment $\times$ trials: $F(12,320) = 0.49$, $p = 0.91$; treatment $\times$ sex $\times$ trials: $F(12,320) = 0.48$, $p = 0.92$] (supp. table 3). The long term memory was not affected by the treatment as no significant effect was observed on the latency to reach the platform on the next day of the test [treatment: $F(3,64) = 0.31$, $p = 0.81$; treatment $\times$ sex: $F(3,64) = 0.41$, $p = 0.74$] (supp. table 3).

There were no differences between lactationally $\sum6$ NDL-PCB-exposed adult mice compared to controls [treatment: $F(3,64) = 0.17$, $p = 0.91$; treatment $\times$ sex: $F(3,64) = 0.09$, $p = 0.96$] (supp. table 3) with regard to the immobility behavior in the TS test, suggesting that the treatment did not alter mice mood.

IV.8. Effects on oxidative status in central and peripheral cells

On PND 14, no significant changes were found in the reactive oxygen species levels in the cerebellar neural cells [$F(3,36) = 0.40$, $p = 0.75$], astrocytes ($H = 1.05$, $p = 0.79$), oligodendrocytes ($H = 1.65$, $p = 0.65$) or microglia ($H = 0.84$, $p = 0.84$) of $\sum6$ NDL-PCB-exposed male mice in comparison to the controls (table 3).

Cells	Control	$\sum6$ NDL-PCBs 1 ng/kg	$\sum6$ NDL-PCBs 10 ng/kg	$\sum6$ NDL-PCBs 100 ng/kg
Neural cells	4.48 ± 0.19	4.19 ± 0.27	4.40 ± 0.17	4.53 ± 0.30
Astrocytes	4.96 (4.65 – 5.30)	5.12 (4.69 – 5.17)	5.00 (4.90 – 5.38)	5.09 (4.96 – 5.32)
Oligodendrocytes	5.54 (5.41 – 5.69)	5.36 (5.32 – 5.66)	5.50 (5.15 – 5.67)	5.23 (5.07 – 5.50)
Microglia	4.60 (3.95 – 5.87)	4.13 (3.36 – 6.24)	4.89 (3.15 – 6.70)	4.44 (3.89 – 6.53)

Table 3. The effects of lactational exposure to $\sum6$ NDL-PCBs compared to the controls on the oxidative status (mean fluorescence intensity: FMI) in the cerebellar neural cells of juvenile male mice on PND 14 (n = 10/group). Data are reported as the mean ± SEM or the median (IQR: 25, 75).

On PND 21, the Kruskal-Wallis tests have shown significant differences between groups in the production of reactive oxygen species in cerebellar neural cells ($H = 9.70$, $p =$

0.02) and neural stem cells ($H = 8.54$, $p = 0.04$). *Post hoc* comparisons have shown a significant induction of oxidative stress in cerebellar cells of the offspring male mice exposed to 1 ng/kg ($z = $ -2.40, $p = 0.02$) and 100 ng/kg ($z = $ -2.08, $p = 0.03$) (table 4). Moreover, a significant increase in reactive oxygen species production was revealed in neural stem cells of mice exposed to ∑6 NDL-PCBs at 1 and 100 ng/kg ($z = $ -2.08, $p = 0.04$; $z = $ -2.00, $p = 0.04$, respectively) (table 4).

Cerebellar cells	Control	∑6 NDL-PCBs 1 ng/kg	∑6 NDL-PCBs 10 ng/kg	∑6 NDL-PCBs 100 ng/kg
Neural cells	1.25 (1.23 – 1.27)	1.29* (1.27 – 1.30)	1.26 (1.25 – 1.27)	1.27* (1.27 – 1.28)
Neural stem cells	1.24 (1.20 – 1.25)	1.26* (1.25 – 1.27)	1.23 (1.21 – 1.25)	1.26* (1.25 – 1.28)

*Table 4. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls on the oxidative status (mean fluorescence intensity: FMI) in the cerebellar neural and neural stem cells of male mice on PND 21 (n = 6/group). Data are reported as the median (IQR: 25, 75). *p < 0.05 significantly different from the control.*

Nevertheless, when we analyzed reactive oxygen species formation in both neural and glial cells in the three brain regions, including cortex, cerebellum, hippocampus of animals on PND 21, we observed significant changes across groups in cortex [$F(3,12) = 15.38$, $p = 0.0002$] and cerebellum [$F(3,12) = 11.94$, $p = 0.0006$] but not in hippocampus [$F(3,12) = 0.77$, $p = 0.53$]. In cerebellum, *post hoc* analyses have confirmed the induction of oxidative stress in the neural and glial cells in lactationally ∑6 PCB-NDL-exposed male mice at 1 and 10 ng/kg ($p = 0.04$ and $p = 0.006$, respectively) when compared to the male controls (fig. 7). Moreover, a trend was observed at 100 ng/kg ($p = 0.06$) (fig. 7). In cortex, lactationally ∑6 NDL-PCB-exposed juvenile male mice at 1, 10 and 100 ng/kg ($p = 0.0007$, $p < 0.0001$, $p = 0.009$, respectively) exhibited a significant increase in reactive oxygen species levels when compared to the controls (fig. 7).
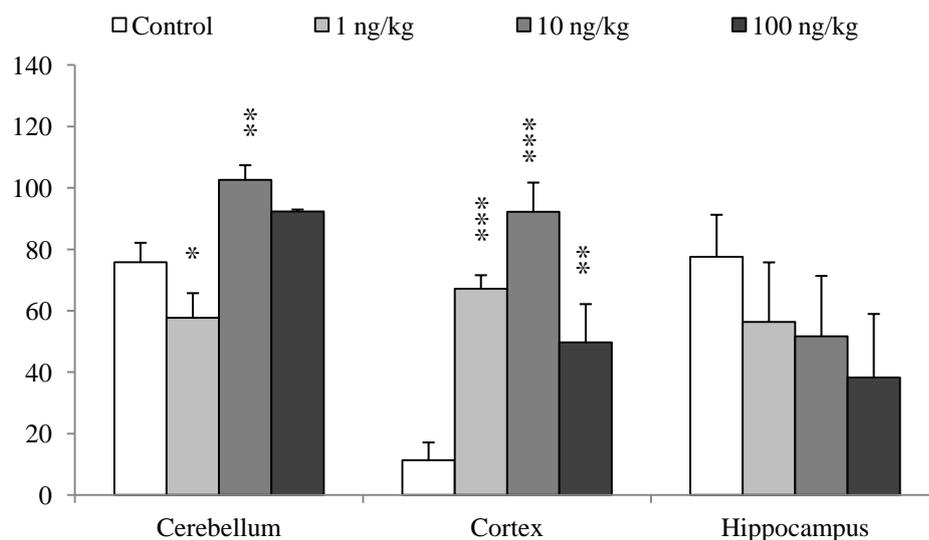
Figure 7. The effects of lactational exposure to ∑6 NDL-PCBs compared to the controls on the oxidative status (mean fluorescence intensity: FMI) in the cerebral neural and glial cells of male mice on PND 21 (n = 4/group). Data are reported as the mean ± SEM. *p < 0.05, **p < 0,01 and ***p < 0,001 significantly different from the controls.

Regarding to peripheral cells, the lactational exposure to ∑6 NDL-PCB did not induce any changes in the production of reactive oxygen species in lymphocytes [$F(3,24) = 0.96$, $p = 0.42$] or in granulocytes ($H = 2.63$, $p = 0.45$) in male mice in comparison to the controls on PND 21 (supp. table 4).

IV.9. Effects on cell death

A decrease in cell viability was observed in neural cells ($H = 11.00$, $p = 0.01$) and in neural stem cells ($H = 10.07$, $p = 0.02$) between male mice groups on PND 21. Moreover, the results have shown a significant lower rate of viable neural cells in mice whose mothers were exposed to 1, 10 and 100 ng/kg ($z = -2.72$, $p = 0.006$; $z = -2.56$, $p = 0.01$; $z = -2.24$, $p = 0.02$, respectively) compared to control mice (table 5). The results have also shown a significant decrease in viable neural stem cells in male mice exposed to PCBs, at the three levels ($z = -2.88$, $p = 0.004$; $z = -1.92$, $p = 0.05$; $z = -2.24$, $p = 0.02$, respectively) (table 5). Similarly, significant differences were observed in the percentage of apoptotic neural cells ($H = 12.19$, $p = 0.01$) and neural stem cells ($H = 9.77$, $p = 0.02$) between groups. Indeed, a significant increase in the percentage of apoptotic neural cells was detected in cerebellar tissue from mice

exposed to $\sum6$ NDL-PCBs at 1 and 10 ng / kg (z = -2.72, p = 0.006; z = -2.56, p = 0.01) (table 5). The Mann-Whitney U-test has also shown a significantly higher rate of apoptotic neural stem cells in mice exposed at 1 and 100 ng/kg compared to control mice (z = -2.88, p = 0.004; z = -2.32, p = 0.02, respectively) (table 5). Early exposure to $\sum6$ NDL-PCBs *via* lactation did not significantly increase the rate of necrotic neural stem cells (H = 2.61, p = 0.46). However, the percentage of necrotic neural cells was significantly increased in groups exposed to $\sum6$ NDL-PCBs at 1 and 100 ng/kg compared to the control group (H = 8.20, p = 0.04; z = -2.24, p = 0.02; z = -2.24, p = 0.02, respectively) (table 5).

Cells	% Cells	Control	$\sum6$ NDL-PCBs 1 ng/kg	$\sum6$ NDL-PCBs 10 ng/kg	$\sum6$ NDL-PCBs 100 ng/kg
Neural cells	Viable	99.42 (99.38 – 99.79)	96.67** (95.51 – 98.45)	97.81** (97.24 – 98.86)	98.22* (96.31 – 99.17)
	Apoptotic	0.07 (0.01 – 0.18)	0.90** (0.66 – 1.18)	1.05** (0.3 – 1.19)	0.34 (0.24 – 0.76)
	Necrotic	0.28 (0.12 – 0.44)	1.42* (0.38 – 2.91)	0.44 (0.31 – 0.86)	0.97* (0.46 – 1.98)
Neural stem cells	Viable	99.63 (99.56 – 99.67)	98.48** (97.96 – 98.91)	97.90* (97.49 – 98.3)	99.22* (98.1 – 99.48)
	Apoptotic	0.06 (0.05 – 0.15)	0.80** (0.49 – 0.96)	0.67 (0.12 – 1.05)	0.34* (0.19 – 0.81)
	Necrotic	0.14 (0.09 – 0.24)	0.26 (0.25 – 0.29)	0.17 (0.04 – 0.27)	0.13 (0.10 – 0.24)

Table 5. The percentage of viability, apoptosis and necrosis in cerebellar neural cells and neural stem cells of juvenile male mice on PND 21 (n = 6/group). Data are reported as the median (IQR: 25, 75). *p < 0,05, **p < 0,01 significantly different from the control.

IV.10. Effects on mRNA expression

When we examined male and female mice together, statistical analyses revealed no significant sex effect, independently of exposure level and brain area. In the cortex and hippocampus, lactational exposure to $\sum6$ NDL-PCBs at all of the doses resulted in no significant effect on GABA$_{A\alpha1}$ (H = 3.00, p = 0.39; H = 1.79, p = 0.62, respectively), RyR$_3$ (H = 0.64, p = 0.88; H = 3.33, p = 0.34, respectively), 5-HT$_{1A}$ (H = 5.59, p = 0.13; H = 2.74, p = 0.43, respectively), or μ-OR$_1$ (H = 1.02, p = 0.79; H = 0.26, p = 0.97, respectively) mRNA expression compared to controls (supp. table 5). Lactational exposure to 10 ng/kg $\sum6$ NDL-PCBs caused a significant increase in RyR$_3$ expression in the cerebellum compared to controls

($H = 9.01$, $p = 0.03$) (fig. 8B). Similarly, there was also a slight increase at 10 ng/kg $\sum6$ NDL-PCBs in GABA$_{A\alpha1}$ expression in the cerebellum, but it was not statistically significant ($H = 6.68$, $p = 0.08$) (fig. 8A). For 5-HT$_{1A}$ and μ-OR$_1$ expression in the cerebellum, no significant effects were observed in the exposed animals ($H = 3.44$, $p = 0.33$; $H = 1.97$, $p = 0.58$, respectively) (fig. 8C and D).
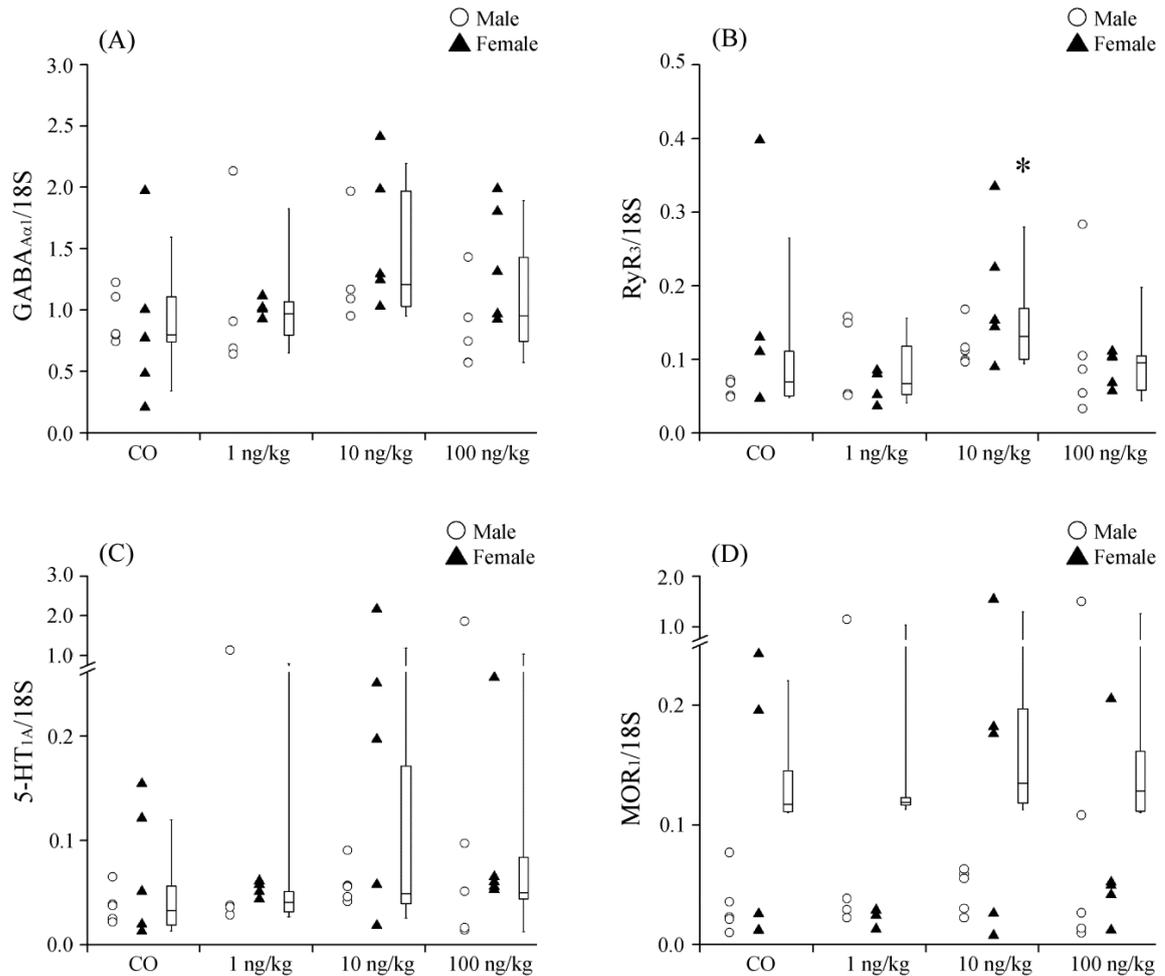
Figure 8. The effects of lactational exposure to $\sum6$ NDL-PCBs compared to the controls (CO) on the mRNA expression of GABA$_{A\alpha1}$, RyR$_3$, 5-HT$_{1A}$, and MOR$_1$ in the cerebellum (A, B, C, D) of juvenile mice (PND 14). The mRNA levels were measured by qPCR and normalized to 18S expression. The result for each animal (four to five per group) was sex-specific. In addition, box plot diagrams of the medians, independent of sex, are shown with the box depicting the range of values from the 25th percentile (lower bar) to the 75th percentile (upper bar). The lines/errors extend to the largest and smallest values. Statistically significant difference (*) between the exposed and control mice.

The Kruskal Wallis tests revealed no significant differences between male mice exposed to $\sum6$ NDL-PCBs and control mice in mRNA expression of Cat ($H = 3.75$, $p = 0.29$),

CuZnSOD$_1$ ($H = 1.63$, $p = 0.65$), Glo$_1$ ($H = 0.86$, $p = 0.83$), GPx$_1$ ($H = 2.67$, $p = 0.44$) or Gsr$_1$ ($H = 2.64$, $p = 0.45$), although a high inter-individual variability was noticed within the same group of mice (fig. 9).
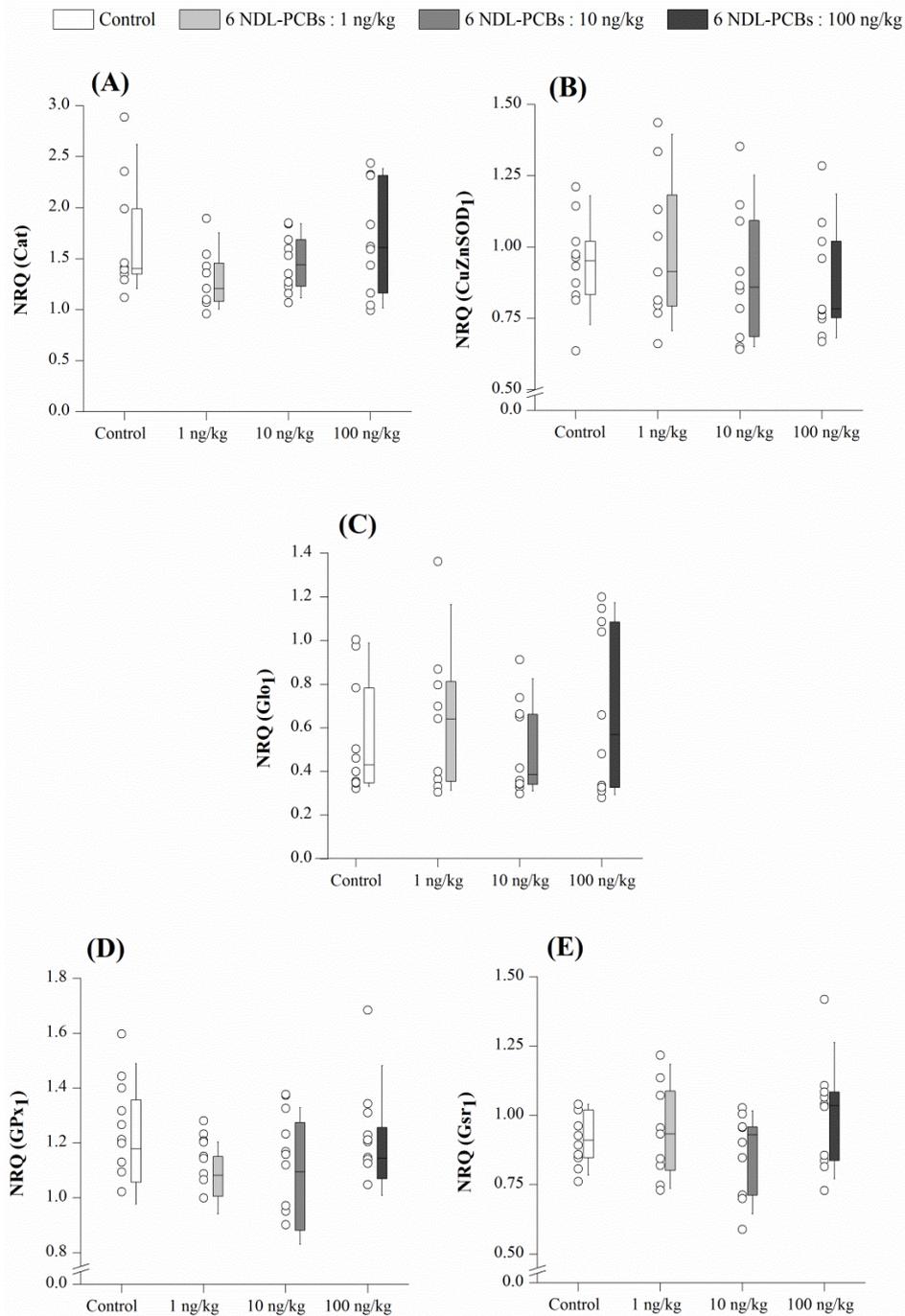
Figure 9. The effects of lactational exposure to ∑6 NDL-PCBs compared to controls (CO) on the mRNA expression of Cat, CuZnSOD₁, Glo₁, GPx₁ and Gsr₁ in the cerebellum (A, B, C, D, E) of juvenile mice (PND 14). The mRNA levels were measured by qPCR and normalized to housekeeping gene expression (normalized relative quantity: NRQ) (n = 10 male/group). In addition, 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). The lines/errors extend to the largest and smallest values.

Microarray analysis revealed that developmental exposure to $\sum 6$ NDL-PCBs (10 ng/kg) induced a statistically significant dysregulation of 1358 genes [$p < 0.4$, fold change (FC) > 2] in neural cells from cerebellar juvenile male mice (PND 14): 693 of these genes were upregulated and 665 were downregulated. The overexpressed genes belong to 6-GO (Gene Ontology, $p < 0.0001$), including cell cycle, DNA replication, cell cycle checkpoint response to DNA damage stimulus, regulation of RNA biosynthetic process and microtubule cytoskeleton organization. Under-regulated genes belong to five GO terms ($p < 0.0001$), including transmission of nerve impulse, projection neuron, synapse hand, cell junction and regulation of RNA biosynthetic process.

Furthermore, no significant differences were detected in the gene expression of Cat, $CuZnSOD_1$, GPx_1, Gsr_1, $\mu\text{-}OR_1$, or $5\text{-}HT_{1A}$ receptors in cerebellar neural cells of mice exposed to $\sum 6$ NDL-PCBs at 10 ng/kg compared to control mice. However, there was a significant decrease in the gene expression of $GABA_{A\alpha 1}$ (FC: 2.9) (fig. 10A) and Glo_1 (FC: 2.9) (fig. 10B) and an increase in RyR_3 (FC: 2.7) (fig. 10C) in neural cells following lactational exposure to $\sum 6$ NDL-PCBs.
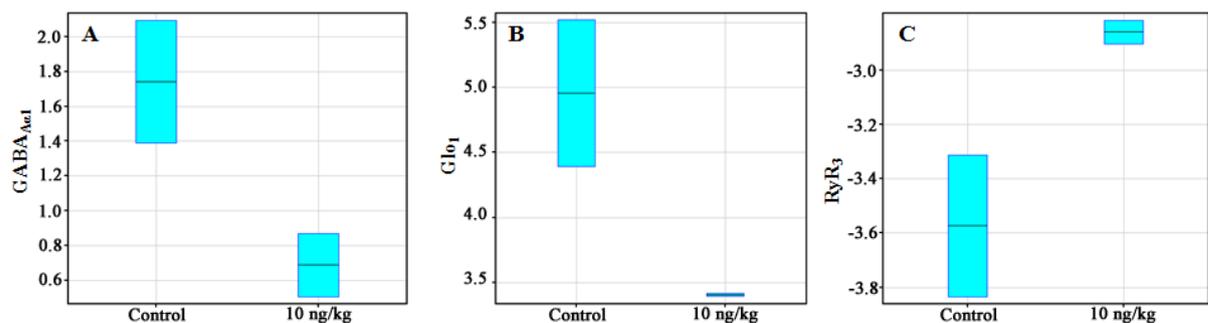
Figure 10. mRNA expression levels of $GABA_{A\alpha 1}$ (A), Glo_1 (B), RyR_3 (C) in neural cells from juvenile male mice lactationally exposed to $\sum 6$ NDL-PCBs at 10 ng/kg and their controls (n = 10/group) as determined by microarray analyses. Results are reported as the median (IQR: 25-75).

In addition, the levels of mRNA encoding the synaptotagmin-10 (syt10) were significantly much higher in the exposed mice than in control mice (FC: 110) (fig. 11A). Moreover, statistical analyses showed that the amount of mRNA of vomeronasal 2 receptor

123 (vmn2r123) was higher in the cerebellar neural cells exposed to $\sum$6 NDL-PCBs (FC: 30) than controls (fig 11B).
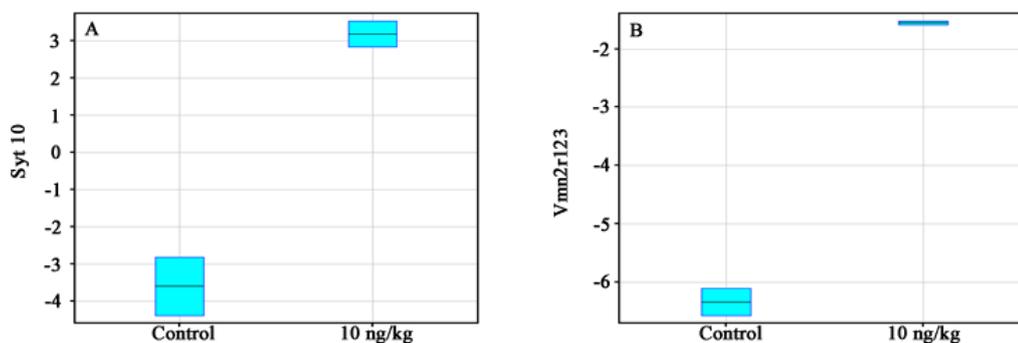
Figure 11. mRNA expression levels of Syt10 (A) and Vmn2r123 (B) in neural cells from juvenile male mice lactationally exposed to $\sum$6 NDL-PCBs at 10 ng/kg and their controls (n = 10/group) as determined by microarray analyses. Results are reported as the median (IQR: 25-75).

V. Discussion

In this study, biomonitoring of the adverse effects of lactational exposure to $\sum$6 NDL-PCBs at low levels on postnatal neurodevelopment, neuronal receptor gene expression, oxidative status, cell death, behavior and cognitive function was performed in neonatal mice (PND 0) and in mice at more advanced life stages (up to PND 275). Our results showed that mice whose mothers were orally exposed to $\sum$6 NDL-PCBs presented significant differences, which were at times sex-dependent, in comparison to mice whose mothers were orally exposed to vehicle. The harmful effects of lactational exposure to $\sum$6 NDL-PCBs were, in some cases, temporary, e.g., the difference in the turning reflex in pups, and in other cases, they were persistent, e.g., the high level of anxiety observed in offspring mice at more progressive life stages, which could be related to the overexpression of the RyR_3 gene in the cerebellum. However, other functions, including memory, learning and mood, as well as changes in the gene expression of neurotransmitter receptors in several brain areas, including 5-HT$_{1A}$, MOR$_1$ and GABA$_{A\alpha1}$, were not affected following lactational exposure to $\sum$6 NDL-PCBs (Elnar et al., 2012).

Human studies have shown that PCBs, which include the six main widespread NDL-PCBs, constitute a health concern for breastfed babies, among other individuals, as they might receive, based on the levels found in breast milk, up to 2.5 mg of $\sum 6$ NDL-PCBs during the first six months of their postnatal development (Angulo et *al.*, 1999; Atkinson, 1988). In fact, the lactational period represents a critical phase of offspring brain development, as it is a sensitive phase for neurodevelopment and neuromaturation. Although its benefits are many (psycho, immunological, nutritional, digestive, metabolic, etc.), breast milk contributes to 5-10 % of the total body burden of PCBs observed in the adult age (Afssa, 2007). Moreover, it has been shown that during this period, the infant intake might be two orders of magnitude higher that adult exposure and receives 2.5 times higher concentrations of NDL-PCBs than adults (EFSA, 2005). The brain presents temporal windows of unique susceptibility to environmental insults that have no counterpart in the mature nervous system (Faroon et *al.*, 2001; Landrigan et *al.*, 2005). Thus, if a developmental process in the brain is altered, there is little potential for subsequent repair, and the consequences can therefore be permanent (Rice and Barone, 2000). In this context, a prospective cohort study of children whose mothers resided near a PCB-contaminated harbor in New Bedford has revealed an association between early exposure to organochlorines, and PCBs in particular, with neuropsychological deficits, including inattentive and impulsive behaviors; however, cord serum PCB analyses indicated low exposure levels of PCBs 118, 138, 153 and 180 (Sagiv et *al.*, 2012). In this epidemiological longitudinal study, the effect of PCBs was sex-specific and affected only boys. Consistent with this finding, several reports have considered prenatal exposure to PCBs as a risk factor for attention deficit/hyperactivity disorder (Eubig et *al.*, 2010; Savig et *al.*, 2010) and poorer response inhibition in children (Stewart et *al.*, 2003).

The results from this study highlight the brain vulnerability of offspring exposed to $\sum 6$ NDL-PCBs through lactation based on the altered behavioral performances recorded during mouse development at early and more advanced life stages, which are more specific for

nervous system dysfunction. For example, female pups whose mothers were treated with 100 ng/kg ∑6 NDL-PCBs required more time to turn their heads upwards compared to controls on PNDs 9 and 11 in the NG test, which evaluates the vestibular function or locomotor coordination; however, no significant differences were found in the males' reflex in the same test (Elnar et *al*., 2012).

Moreover, in the FGS test, muscular strength of mice was not affected by the ∑6 NDL-PCBs on PNDs 9 and 11, while female and male animals whose mothers were exposed to ∑6 NDL-PCBs at 10 and 100 ng/kg respectively showed a significant weight gain compared to control mice. This effect was transient and disappeared within the end of mice weaning (PNDs 16 and 20). In addition, a significant decrease was observed in the general activity of juvenile (PND 28) male mice exposed to 1 and 10 ng/kg NDL-PCBs in the OF test. However, the effects of postnatal exposure to ∑6 NDL-PCBs on motor activity disappeared with age, as no significant differences between the mice groups were observed on PND 40 and PND 160, using the number of entries into closed arms of the EPM test and the locomotion into the dark box of the LDB test as markers of locomotor activity. Nevertheless, the exploratory behavior of the mice postnatally exposed to ∑6 NDL-PCBs *via* lactation in the aversive parts of the EPM test (the open arms) and the LDB test (the lit box) were significantly diminished compared to controls, indicating an increase in anxiety-related behavior. The effects of developmental exposure to ∑6 NDL-PCBs at all doses on anxiety were sex-independent and permanent, as revealed by two different and well-known tests of anxiety that were performed separately during distinct developmental periods (PNDs 40 and 160). The high level of anxiety could partially explain the significant increase in the pole grasping latency of male animals in the WESPOC test, as swimming in the aqueous medium during this assay could be considered a stressful situation. However, a lack of visuomotor integration should not be ruled out. Interestingly, during this investigation, maternal behavior was unaffected following daily oral exposure to a PCB mixture, as revealed by nest-building activity and a retrieval test.

Therefore, the relationship between the set of behavioral disturbances observed in mice whose mothers were exposed to $\sum$6 NDL-PCBs and maternal behavior is excluded.

Consistent with our results, several studies have emphasized the neurobehavioral toxicity of PCBs including non-dioxin-like congeners, particularly during early exposure; however, there are numerous differences in study design, the use of congeners, and dosage (which were 10- to 1000-fold higher than the levels used in our study), among others variables. In addition to PCB concentration, the mixed congener profile plays a role in the toxicity of the observed panel. Indeed, most studies performed on animal models have assessed the adverse neurodevelopmental effects of individual PCB congeners or commercial PCB mixtures. However, the mixed PCB profile used in this study closely mimics human PCB exposure from fish, which is different from that of commercial PCB mixtures (Kostyniak et al., 2005).

For instance, Aroclor 1254 (40 mg/kg) and reconstituted mixture based on the profile of PCB congeners in breast milk, which were administrated to the rats during gestational and lactational periods, induced a significant decrease in the body weight of the offspring starting from parturition until adulthood (Hany et al., 1999). In addition, a significant body weight loss has been noticed at birth and weaning in the offspring male mice whose mothers were gestationally and lactationally exposed to individual PCB 28 (8 and 32 mg/kg), PCB 118 (4 and 16 mg/kg) and PCB 153 (64 mg/kg), but no difference between groups was noticed when animals became adults (Schantz et al., 1995). It has been shown that perinatal exposure to a commercial PCB mixture (Aroclor 1254) at doses of 26 mg/kg (Overmann et al., 1987), 10 mg/kg (Nguon et al., 2005), 6-18 mg/kg and 54 mg/kg (Sugawara et al., 2006) impaired the turning reflex of rodent pups in the NG test. Moreover, at 18 mg/kg, Sugawara et al. (2006) found that PCB-exposed young mice significantly decreased their walking speed in the OF test while a significant increase in the motor activity was reported in offspring mice following perinatal exposure to PCBs at 11 and 82 mg/kg (Storm et al., 1981) and following lactational

and postnatal exposure to PCBs at 6 and 18 mg/kg (Tian et *al*., 2011). Nevertheless, a transient hypoactivity was observed in two weeks old offspring rats whose mothers were exposed to Aroclor 1254 at 4 and 8 mg/kg from gestational day (GD) 6 until PND 21 (Goldey et *al*., 1995, Goldey and Crofton, 1998). In addition, no significant effect was observed on muscular strength in juvenile Aroclor 1254-exposed rats at 1 and 6 mg/kg during gestational and lactational periods (Bushnell et *al*., 2002). However, although *in utero* exposure to PCB 95 at 8 and 32 mg/kg induced a reduction in the locomotor activity of adult rats on PND 100, this effect was not significant when animals were younger (Schantz et *al*., 1997). Eriksson and Fredriksson (1996) also revealed that subacute neonatal exposure to single NDL-PCB congeners, including PCB 28 (3.6 mg/kg) and PCB 52 (4.1 mg/kg), induced a decrease in the motor activity in adult mice. In contrast, Boix et *al*. (2011) showed that PCB 52 (1 mg/kg) did not affect motor activity in gestationally/lactationally exposed rats. In addition, they found that PCB 138 reduced motor activity in mice in a sex-independent manner, unlike PCB 180, which altered this function only in male mice. Furthermore, rats postnatally exposed to PCB 153 exhibited hyperactive behavior (Holene et *al*., 1998), which was also provoked in mice with brain levels of 20 µg/kg (Eriksson, 2007). It has also been shown that gestational and lactational exposure to a 10 mg/kg mixture of PCBs (PCBs 126, 138, 153 and 180) in rats did not affect locomotor activity or anxiety behaviors (Colciago et *al*., 2009).

Thus, these variables and conflicting results could be related to several factors, e.g., the form of PCBs used (i.e., individual PCB congeners or mixtures), which could substantially influence their mechanism of action on specific cellular pathways (i.e., xenobiotics in mixtures, contrary to individual conditions, function additively, synergistically or even antagonistically, resulting in specific responses that vary based on the complexity of the mixtures). The toxicity of NDL-PCBs also depends on differences in their metabolism, absorption and excretion, as less chlorinated congeners such as PCBs 52 and 101 are more easily metabolized and excreted than the more persistent ones (PCBs 138, 153 and 180) (van

Larebeke et *al.*, 2001). The concentration of PCBs could also play a role in the disparity in the observed behavioral alterations. For example, POPs act on different neuronal functions (e.g., reflex, mood, anxiety, cognitive performances) with different thresholds of sensitivity, which may explain their negative dose–response relationship (Bouayed et *al.*, 2009a,b and 2012).

Many studies have shown that PCBs are involved in the alteration of neurotransmitter-gated ion channels, such as those related to dopamine, GABA, and serotonin, and receptors such as RyR (Boix et *al.*, 2011; Fernandes et *al.*, 2010; Pessah et *al.*, 2010), which have been implicated in many behavioral and cognitive functions (Schantz et *al.*, 1997). Nevertheless, there has been a lack of studies evaluating changes in the mRNA gene expression of central receptors in the juvenile mice induced by lactational exposure to a mixture of NDL-PCBs, although some studies have linked behavioral results to mRNA expression measurements (Bouayed et *al.*, 2009a, 2012; Hovatta et *al.*, 2005). The proposed mechanisms of action of NDL-PCB compounds or their metabolites on the central nervous system generally involve processes that affect intracellular signaling and neurotransmitter systems (Kodavanti, 2005). Alterations in these proposed pathways could lead to several neurobiological alterations, including changes in the gene expression of several transcription factors, resulting in developmental disturbances and neurotoxic responses such as motor dysfunction, anxious behavior, and learning and memory deficits (Schantz et *al.*, 1997).

It was noticed that lactational and postnatal exposure to Aroclor 1254 (6 and 18 mg/kg) induced an anxiolytic-like effect following the performances of female offspring mice in the OF and the EPM tests (Tian et *al.*, 2011). Piedrafita et *al.* (2008) showed that developmental exposure to PCB 153 at high levels induced temporary learning deficits in young mice, but the effect was not persistent in adult animals. Moreover, 8-week-old Aroclor 1254-exposed offspring mice exhibited learning and memory deficits in the same test (Sugawara et *al.*, 2006). Colciago et *al.* (2009) have also demonstrated that PCBs did not affect either memory retention or mood in rats. Consistent with our results, no aberrations were observed at more

advanced life stages (PND 268) in the cognitive function of mice whose mothers were orally exposed to ∑6 NDL-PCBs compared to controls, as revealed by the MWM test. Their mood was also unaffected, as revealed by the TS test on PND 275. Similarly, the mRNA gene expression of 5-HT_{1A}, $\mu\text{-OR}_1$ and $GABA_{A\alpha1}$ did not show any alterations in the hippocampus, cerebellum or cortex of juvenile mice compared to controls (Elnar et *al*., 2012).

Intriguingly, significantly higher expression of RyR_3 in the cerebellum of the juvenile male and female mice that were lactationally exposed to 10 ng/kg was observed, despite the well-recognized high individual variation in sensitivity to PCBs (Curran et *al*., 2011; Elnar et *al*., 2012). RyRs, a family of intracellular calcium release channels, are widely expressed in the mammalian brain. Specifically, the third isoform (RyR_3) plays an important role in the regulation of intracellular calcium (Ca^{2+}) homeostasis (Giannini et *al*., 1995). RyR_3 is not equally distributed across the structures of the mouse brain; it is described to be highly expressed in the granular layer of the cerebellum (Giannini et *al*., 1995). Dysregulation of cerebellar RyR isoform expression has been correlated to behavioral deficits in the brains of PCB-exposed rats due to changes in intracellular signaling within the cerebellum (Roegge et *al*., 2006). Pessah et *al*., (2010) described a detailed structure-activity relationship between PCBs and RyR, suggesting the involvement of these receptors in NDL-PCB neurotoxicity and supporting the hypothesis that NDL-PCBs may disrupt normal patterns of neuronal connectivity *via* effects on dendritic growth and plasticity. Recent studies have implicated the involvement of the cerebellum in many behavioral disturbances, including anxiety-related behavior (Richter et *al*., 2005). It has also been suggested that RyR_3 plays a role in the anxiety/fear response, as RyR_3-deficient mice exhibit impairments in the fear response in the EPM test (Kouzu et *al*., 2000). Consequently, overexpression of RyR_3 in the cerebellum following lactational exposure to ∑6 NDL-PCBs (10 ng/kg) is a potential neurobehavioral NDL-PCB toxicity mechanism that may mediate the persistent anxiety-like behavior observed in adult mice, although other pathways and non-investigated brain receptors, such as other 5-

HT and GABA receptor subtypes (e.g., 5-HT_{1B}, 5-HT_{2C} and $GABA_{A\alpha 2}$), could be involved. Oxidative stress may also be a putative mechanism of NDL-PCB congener action, as this cytotoxic condition has been linked to several behavioral disturbances, including anxiety (Bouayed et *al*., 2009c).

The hypothesis is that PCBs affect the oxidative stress caused by the elevation of intracellular calcium and/or activation of neurotransmitter receptors (Fonnum and Mariussen, 2009). Thus, several routes are possible, through the disruption of calcium homeostasis of mitochondria, the activation of RyR, inositol triphosphate (IP_3), N-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA), protein kinase C (PKC), phospholipase A_2 (PLA_2), and nitric oxide synthase (NOS) followed by the formation of nitric oxide ($NO^{\bullet}$). PCBs may also inhibit the vesicular transport of monoamines (VMAT) and cause auto-oxidation of dopamine followed by the formation of $O_2^{\bullet-}$. This can also lead to the formation of reactive oxygen species and activation of NOS and PLA_{2-} and protein kinases ERK (extracellular signal-regulated kinases) (Fonnum et Mariussen, 2009).

Various *in vivo* and *in vitro* studies have highlighted the involvement of NDL-PCBs in the alteration of the levels of intracellular calcium (Ca^{2+}), neurotransmission, gene expression, the induction of the formation of reactive oxygen species and reduction of cell viability in several brain regions (Carrasco and Hidalgo, 2006; Fonnum and Mariussen, 2009; Orrenius et *al*., 2011; Pessah et *al*., 2010; Venkataraman et *al*., 2007; Westerink, 2006). Langeveld et *al*. (2012) showed a significant high increase in the basal concentration of Ca^{2+} and an inhibition of calcium channel voltage-dependent (CCVD) when PC12 cells (cells derived from rat pheochromocytoma) were exposed to PCBs 53 and 95 (0,1 μM) and to PCBs 47, 51, 52, 53, 95 and 104 (1 μM). With the exception of PCB 136, hexa-and hepta-chlorinated congeners did not affect the depolarization of Ca^{2+}. Moreover, previous studies have shown that some congeners of NDL-PCBs, including PCBs 19, 28, 47, 51, 52, 95 and 100 act as agonists of postsynaptic $GABA_A$ receptors in humans and that GABA neurotransmission depends on the

chlorination and concentration of NDL-PCBs used (Antunes Fernandes et *al*., 2010a,b). For example, potentiation and activation of $GABA_A$ receptor are limited to NDL-PCBs that have 3 to 5 chlorine atoms, 1 to 3 *ortho*-substitutions, an equal number of *meta-* substitutions on benzene rings and non-adjacent *para- meta-* substitutions on the same benzene ring. Thus, the PCB congener 47 seems to be the most potent full agonist of the neurotransmitter GABA and the antagonist of nicotinic acetylcholine (nACh) (Hendriks et *al*., 2010). In the case of mixtures, the association between PCB 47 and the hydroxylated metabolite of BDE-47, 6-OH-BDE-47, induces an additive activation and potentiation of $GABA_A$ receptors, and an additive inhibition of nACh receptors. In addition, Howard et *al*. (2003) showed that the activity of PCBs is inhibited by antagonists of RyR and α-tocopherol in hippocampal neurons in rats, indicating a link between PCB exposure and induction of intracellular calcium, apoptosis and oxidative stress. *In vitro* studies conducted in rats have shown that NDL-PCB congeners, including PCB 153 (25 and 50 microM) induced oxidative stress in cerebellar granule cells by initiating the release of calcium from intracellular storage sites *via* RyR (Mariussen et *al*., 2002). Moreover, an increase in the production of reactive oxygen species in synaptosomes following exposure to individual congeners PCBs 1, 4, 11 and 19 (12.5 μM) (Voie and Fonnum, 2000) and in cerebellar granule cells exposed to PCB 153 (25 and 50 μM) (Mariussen et *al*., 2002) were observed in young rats. A reduction of viability has been reported in cerebellar granule cells from juvenile rats exposed to PCB 153 and Aroclor 1254 (6.25, 12.5, 25 and 50 μM) (Mariussen et *al*., 2002). Costa et *al*. (2007) have shown that PCB 153 did not increase the percentage of cell death in the central nervous system of mice at gestational day (GD) 21. Roegge et *al*. (2006) have revealed that perinatal exposure to Aroclor 1254 (6 mg/kg) did not affect the structure of Purkinje cells in young offspring rats (PND 21). However, few studies have evaluated the neurotoxic effects induced by developmental exposure to NDL-PCBs on oxidative stress and cell death *ex vivo* in mice.

In this study, we have shown that the $\sum 6$ NDL-PCB induces oxidative stress depending on mice postnatal age and on selected cell type. For example, on PND 14, the levels of reactive oxygen species in isolated cerebellar cells, such as astrocytes, neurons, oligodendrocytes and microglia were not significantly different from the control mice, while significant increase in reactive oxygen species formation was observed on PND 21 in cerebellar neurons and neural stem cells of lactationally exposed male mice at 1 and 100 ng/kg. When we examined reactive oxygen species levels in the three brain regions on PND 21, we noticed that the mean fluorescence intensity in cortex (1-100 ng/kg) and in cerebellum (1 and 10 ng/kg) of lactationally $\sum 6$ NDL-PCB-exposed mice were significantly different from control mice values, indicating an induction of oxidative stress in these two brain parts while hippocampus was not affected by $\sum 6$ NDL-PCB postnatal exposure. In addition, lactational exposure to $\sum 6$ NDL-PCBs induced a significant decrease in the percentage of apoptotic and necrotic neural and neural stem cells collected from the cerebellum of young mice on PND 21 compared to the controls.

On the other hand, we have noticed that developmental exposure to $\sum 6$ NDL-PCBs on PND 14 did not alter the mRNA expression of five antioxidant enzymes from cerebellum of male mice, including Cat, Glo_1, GPx_1, Gsr_1 or $CuZnSOD_1$. However, studies conducted in rats, intraperitoneally exposed to Aroclor 1254 (2 mg/kg) during 30 days, showed reduced levels of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in the cerebellum, cortex, hippocampus (Venkataraman et *al*., 2007) and hypothalamus (Muthuvel et *al*., 2006). Venkataraman et *al*. (2007) also found a reduction in the level of hydrogen peroxide and an increase in lipid peroxidation as well as a depletion of glutathione levels in PCB-exposed rats. In addition, Lee and Opanashuk (2004) found an induction of oxidative stress and changes in protein expression of MnSOD and CuZnSOD following exposure to Aroclor 1254 (1-20 ppm) *in vitro*.

The effect of Aroclor 1254 on gene regulation and protein expression was well investigated by the team Kodavanti. It was shown that exposure to Aroclor 1254 during the perinatal period (GD 6 to PND 21) inhibits the activity of DNA-binding transcription factors, including Sp1 (specificity protein 1), AP1 (activator protein 1), and NF-κB (nuclear factor-kappa B) in the cerebellum and hippocampus in the rat on PNDs 7 and 14 (Basha et *al*., 2006). The analysis conducted by Royland and Kodavanti (2008) suggests that pathways related to calcium homeostasis, intracellular signaling, axon guidance, signaling of the aryl hydrocarbon and transcripts involved in cell proliferation and differentiation and in the development of the nervous system have been significantly altered following exposure to Aroclor 1254. The results obtained from recent works conducted by this team revealed the role of this commercial mixture in the expression of two and eighteen differential proteins in the cerebellum and hippocampus, respectively. These proteins are related to mitochondrial energy metabolism, calcium signaling and nervous system growth (Kodavanti et *al*., 2011). However, Royland and Kodavanti (2008) have highlighted the potential modes of action of developmental Aroclor 1254 exposure on neurochemical (Kodavanti, 2004, 2005, Kodavanti and Tilson, 2000; Seegal, 1996; Morse et *al*., 1996a,b), genomic (Kaya et *al*., 2002; Lein et *al*., 2007; Royland and Kodavanti, 2008; Zoeller et *al*., 2000) and proteomic changes (Gillardin et *al*., 2009; Kodavanti et *al*., 2011) leading to structural (Lein et *al*., 2007) and functional alterations (Kodavanti and Tilson, 1998; Vreugdenhil et *al*., 2004; Widholm et *al*., 2004).

In this study, the transcriptomic analyses conducted in neural isolated cells from lactationally $\sum 6$ NDL-PCB-exposed male mice (10 ng/kg) on PND 14 have shown overexpression of certain genes involved in the repair and response to DNA damage. Significant decreases in the transcription of genes involved in establishing the neuronal projections, the action potential and the number of synapses were also observed in the exposed neurons. Consistent with these results, microtubule reorganization was detected in

these damaged neurons, indicating that loss of connectivity would logically be associated with changes in the establishment of the neuronal cytoskeleton. We also found that there were no significant differences in gene expression of Cat, $CuZnSOD_1$, GPx_1, Gsr_1, $\mu\text{-}OR_1$, $5\text{-}HT_{1A}$ in neural cells isolated from the cerebellum of mice exposed to $\sum6$ NDL-PCBs at 10 ng/kg compared to control mice. These results and the overexpression of RyR_3 appear to converge with analyses obtained previously by qPCR in the whole cerebellum of mice lactationally exposed to $\sum6$ NDL-PCBs at 10 ng/kg (Elnar et *al*., 2012). However, a significant decrease in the mRNA expression of Glo_1 and $GABA_{A\alpha1}$ was observed in neural cells collected from young mice exposed to $\sum6$ NDL-PCBs compared to control mice. This finding was also obtained in the previous qPCR results in the whole cerebellum but statistical analyses revealed no significant differences between the exposed and control groups (Elnar et *al*., 2012). Furthermore, the expression of synaptotagmin-10 (syt10), a protein involved in exocytosis (Cao et *al*., 2011), was significantly increased in neural cells isolated from $\sum6$ NDL-PCB-exposed mice compared to control cells isolated from control mice. $\sum6$ NDL-PCBs has also induced overexpression of vomeronasal 2 receptor 123 (vmn2r123), a protein involved in the social and reproductive behavior (Ryba and Tirindelli, 1997). These two proteins are necessary for the survival and maintenance of the neuron and are involved in the regulation of intracellular calcium signaling which is consistent with several hypothesis and *in vitro* studies mentioned previously (Fonnum and Mariussen, 2009; Kodavanti et *al*., 2011).

In conclusion, we evaluated the adverse effects of developmental exposure to a mixture of NDL-PCBs at doses that are similar to the levels found in contaminated fish matrices using a mouse model for the lactational transfer of PCBs in this study. Although our results cannot be completely extrapolated to humans, regular consumption of contaminated fish matrices by lactating women could affect the optimal development of their newborns.

Results concerning the dosage of the $\sum 6$ NDL-PCBs in the tissues of exposed dams and their offspring (plasma, liver and brain) are being analyzed. Future investigations about the potential toxic effects induced by perinatal exposure to contaminated fish such as eels on the offspring central nervous system are planned. Moreover, based on the results of this study related to developmental disorders that were sometimes sex-specific, vulnerability of the endocrine system (Langer et *al*., 2007) and thyroid system (Crofton et *al*., 2000; Kokkinos et *al*., 2007) following early exposure to PCB are warranted.

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VII. Supplementary data

Supplementary table 1. Additional information for qPCR

Gene	Gene bank accession number	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'	Amplicon size (bp)	Amplification Efficiency Mean (%)	Efficiency Range (%)	r^2 Mean	r^2 Range	Slope Mean	Slope Range	Annealing temperature
$GABA_{A\alpha1}$	NM_010250	GACTGCTGGACG GTTATGACAA	AATGTCGGTCTTCAC TTCAGTTACAC	73	98.27	92.1 – 102.7	0.9965	0.995 – 0.998	-3.37	(-3.53) – (-3.26)	61°C
$5\text{-}HT_{1A}$	NM_008308	TCGCTCACTTGG CTCATTGGCTTT	TTCCAACTTCTTGAC CGTCTTGCG	240	97.92	94.5 – 103.7	0.9925	0.983 – 0.997	-3.38	(-3.46) – (-3.24)	61°C
MOR_1	NM_001039652	GCCCTCTATTCT ATCGTGTGTGTA	GTTCCCATCAGG TAGTTAACACTC	186	92.57	90.6 – 94.5	0.9952	0.992 – 0.998	-3.51	(-3.57) – (-3.46)	60°C
RyR_3	NM_177652	ACAGCCCCGCCC CTCTGGAT	TGGCTGCCACCA CCCTTGCT	142	102.30	99.1 – 104.8	0.9948	0.991 – 0.998	-3.27	(-3.34) – (-3.21)	63°C
18S	NR_003278	CGGACAGGA TTGACAGATTG	CAAATCGCTCCA CCAACTAA	83	99.80	99.1 – 100.4	0.9963	0.995 – 0.997	-3.33	(-3.34) – (-3.32)	58°C
Cyc	NM_008907	GGCCGATG ACGAGCCC	TGTCTTTGGAAC TTTGTCTGC	74	-	-	-	-	-	-	58°C
Cat	NM_009804	TGCTTCAGGGC CGCCTTTTTG	GCACATGGGG CCATCACGCT	131	97.21		0.994		-3.39		60°C
$CuZnSOD_1$	NM_011434	GGCGATGAAA GCGGTGTGCG	TTCACCGCTTG CCTTCTGCTCG	82	100.67		0.995		-3.31		60°C
Glo_1	NM_001113560	TCTACTGCAG CAAACGATGC	CTTCTGCAGG AGGGTCAGTC	91	88.18		0.996		-3.64		60°C
GPx_1	NM_008160	TCGGACACCAG GAGAATGGCAA	GAGCGCAGTG GGGTCGTCAC	188	84.65		0.996		-3.75		60°C
Gsr_1	NM_010344	CCGCGATCTC CACAGCAATG	ACCGCTCCACA CATCCTGATTG	169	85.66		0.994		-3.72		60°C
Actb	NM_007393	CACCCGCGAG CACAGCTTCTT	TTTGCACATGC CGGAGCCGTT	118	88.17		0.999		-3.64		60°C
Aip	NM_016666	TGCTGCCGTGT GGAATGCCC	GCCCGCAGCT CTCTGCTTACC	99	90.78		0.999		-3.56		60°C
$Cxxc_1$	NM_028868	GGCAGAAGTG CCGGCTTCGT	TCACCGGCGA GAGCGAGGAA	81	98.81		0.998		-3.35		60°C
Gapdh	NM_008084	CCCCAGCAAGG ACACTGAGCAAG	TGGGGGTCTGGG ATGGAAATTGTGA	91	99.29		0.995		-3.34		60°C
Mrpl48	NM_198831	GTCCACCGCCT CTGCAACCG	TGCCTTGGT CTGGCAGTCGC	97	98.89		0.996		-3.35		60°C
RpolyII	NM_009089	GCTTCCTGGTG AAGACAATG	GGCCAGGAT GTCCTTAATCT	101	97.62		0.991		-3.38		60°C

r^2, correlation coefficient of the slope of the standard curve

Supplementary table 2. Body weight evolution [g], retrieving time [s] and nest quality scores of dam mice exposed to 1, 10 and 100 ng/kg Σ6 NDL-PCBs compared to the controls (n = 10/group).

Test	PND	Control	Σ 6 NDL-PCBs 1 ng/kg	Σ 6 NDL-PCBs 10 ng/kg	Σ 6 NDL-PCBs 100 ng/kg
Body weight evolution (g)	0	40.62 ± 0.46	39.35 ± 0.41	40.21 ± 1.08	40.13 ± 0.81
	4	42.87 ± 0.51	41.25 ± 0.79	42.04 ± 0.66	42.41 ± 0.79
	8	44.23 ± 0.59	43.89 ± 0.63	44.52 ± 0.86	45.00 ± 0.90
	12	45.30 ± 0.70	45.74 ± 0.72	46.84 ± 0.89	45.60 ± 0.72
	16	43.60 ± 0.45	43.88 ± 0.44	44.73 ± 0.99	43.27 ± 0.51
	20	38.84 ± 0.56	37.15 ± 0.56	37.15 ± 0.78	38.31 ± 0.92
Retrieving time (s)	1	56.10 ± 10.67	33.30 ± 5.16	57.20 ± 14.73	52.20 ± 17.45
	5	42.00 ± 15.76	37.40 ± 11.14	27.80 ± 4.52	45.90 ± 21.47
Nest quality scores	0	2.90 ± 0.10	3.00 ± 0.00	3.00 ± 0.00	2.90 ± 0.10
	4	2.80 ± 0.13	2.40 ± 0.16	2.40 ± 0.16	2.50 ± 0.17
	8	2.50 ± 0.17	2.80 ± 0.13	2.40 ± 0.16	2.30 ± 0.26

Data are reported as the mean ± SEM.

Supplementary table 3. The performances of offspring mice in forelimb grip strength (FGS), open field (OF), Morris water maze (MWM) and tail suspension (TS) tests (n = 20/group).

Test	Variables	PND	Control	∑ 6 NDL-PCBs 1 ng/kg	∑ 6 NDL-PCBs 10 ng/kg	∑ 6 NDL-PCBs 100 ng/kg
FGS	Suspension time	9	5.25 ± 0.98	4.40 ± 0.89	4.10 ± 0.61	5.53 ± 0.91
		11	6.70 ± 1.11	6.95 ± 0.99	9.80 ± 2.83	8.21 ± 1.75
OF	Rearing	28	21.70 ± 1.75	16.50 ± 2.02	19.75 ± 1.88	20.10 ± 2.25
MWM	Latency (n = 5 trials)	268	101.12 ± 9.50	112.11 ± 5.01	105.53 ± 6.60	104.75 ± 7.52
			98.71 ± 7.92	95.11 ± 9.71	96.76 ± 9.68	101.45 ± 7.38
			89.71 ± 10.49	85.33 ± 11.08	92.60 ± 10.58	91.45 ± 10.19
			97.06 ± 8.88	80.67 ± 11.24	84.18 ± 11.33	76.25 ± 11.15
			70.29 ± 11.59	88.11 ± 11.18	75.71 ± 12.12	84.35 ± 7.85
	Memory retention	269	58.41 ± 12.33	71.78 ± 10.18	61.41 ± 11.61	59.05 ± 10.48
TS	Immobility time	275	126.82 ± 11.52	132.83 ± 11.36	136.76 ± 12.45	127.55 ± 13.79

Data are reported as the mean ± SEM. Time is expressed in seconds.

Supplementary table 4. The effects of lactational exposure to Σ6 NDL-PCBs compared to controls on oxidative status (arbitrary units) in lymphocytes and granulocytes of juvenile male mice compared to the controls (n = 7/group)

Blood cells	Control	∑6 NDL-PCBs 1 ng/kg	∑6 NDL-PCBs 10 ng/kg	∑6 NDL-PCBs 100 ng/kg
Lymphocytes	8.89 ± 1.80	10.31 ± 1.37	7.64 ± 0.59	9.93 ± 0.73
Granulocytes	51.24 (36.05 – 68.86)	46.04 (42.33 – 65.86)	62.44 (53.39 – 67.99)	68.78 (47.36 – 77.88)

Data are reported as the mean ± SEM or the median (IQR: 25-75).

Supplementary table 5. The effects of lactational exposure to Σ6 NDL-PCBs compared to controls on mRNA expression of GABA$_{Aα1}$, RyR$_3$, 5-HT$_{1A}$, and MOR$_1$ in the cortex and hippocampus of juvenile mice (PND 14). mRNA levels were measured using qPCR and normalized to 18S expression (n = 9-10/group).

Brain area	Normalized genes	Control	∑6 NDL-PCBs 1 ng/kg	∑6 NDL-PCBs 10 ng/kg	∑6 NDL-PCBs 100 ng/kg
Cortex	GABA$_{Aα1}$/18S	0.67 (0.55 – 0.82)	0.59 (0.51 – 0.89)	0.57 (0.49 – 0.72)	0.65 (0.54 – 0.77)
	RyR$_3$/18S	0.11 (0.07 – 0.20)	0.13 (0.06 – 0.17)	0.10 (0.07 – 0.18)	0.12 (0.07 – 0.20)
	5-HT$_{1A}$/18S	0.25 (0.20 – 0.57)	0.24 (0.18 – 0.47)	0.24 (0.15 – 0.47)	0.34 (0.26 – 0.55)
	MOR$_1$/18S	0.09 (0.06 – 0.18)	0.09 (0.06 – 0.16)	0.09 (0.06 – 0.16)	0.10 (0.06 – 0.19)
Hippocampus	GABA$_{Aα1}$/18S	0.47 (0.37 – 0.69)	0.47 (0.37 – 0.63)	0.51 (0.38 – 1.01)	0.42 (0.30 – 0.93)
	RyR$_3$/18S	1.35 (1.22 – 1.38)	1.36 (1.27 – 1.85)	1.38 (1.29 – 1.79)	1.35 (1.13 – 1.89)
	5-HT$_{1A}$/18S	0.47 (0.35 – 0.77)	0.55 (0.35 – 0.82)	0.67 (0.36 – 1.03)	0.65 (0.37 – 0.97)
	MOR$_1$/18S	0.32 (0.25 – 0.51)	0.32 (0.26 – 0.42)	0.33 (0.24 – 0.83)	0.34 (0.26 – 0.63)

Data are represented as medians (IQR: 25-75)

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EDUCATION

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Research project:
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Research project :
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2009-2012   PhD student-Université de Lorraine- Metz, France and Universität des Saarlandes- Saarbrücken, Germany.
Research project: Short and long term neurotoxic effects following lactational exposure to the sum of the six non-dioxin-like polychlorinated biphenyls ($\sum$6 NDL-PCBs) at low levels in offspring mice.

LECTURES AND COURSES

2009-2010   Exercises in neuroscience (vacataire CNU 69) - Sciences Humaines et Arts – Université de Lorraine, Metz, France.
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2010-2011   Lectures and exercises in neuroscience (Attaché Temporaire d'Enseignement et de Recherche (ATER): CNU 69) **-** Sciences Humaines et Arts – Université de Lorraine, Metz, France.
For students of psychology (the first, second and third term)

2011-2012   Lectures and exercises in neuroscience (ATER: CNU 69) - Faculté des sciences – Université Henri-Poincaré, Nancy, France
For students of biology (the first, third and fourth term)

SCIENTIFIC OUTPUT

Posters
2009   Congrès Annuel de la Société Française de Toxicologie, 19-20 Octobre, Nancy-France **:**

- Lactational transfer of benzo[α]pyrène (B[α]P): impact on postnatal neurodevelopment, neuronal receptor gene expression and behavior in Swiss mice. Soulimani R., Bouayed J., Desor F., Ardzivian A., Rychen G.
- Increased aggressiveness and sexual behavior impairment in Swiss mice exposed to a subacute oral exposure to benzo(α)pyrène (B[α]P). Soulimani R., Bouayed J., Desor F., Ardzivian A., Rychen G.

2012	Séminaire Annuel RP2E 19 janvier 2012, Nancy, France
	• Evaluation des effets neurotoxiques induits chez la souris Swiss par exposition de la mère allaitante à un mélange de 6 PCB-NDL. Ardzivian-El Nar A., Desor F., Soulimani R.

Oral communication

2009	International Conference "Frontiers in Environmental Health, Late Consequences of Early Life Exposure?" 28 Octobre, Belvaux-Luxembourg
	• Methodological study for the assessment of behavioural, cognitive and post development disorders induced by chronic exposure to low doses of B[α]P-contaminated diet in newborn mice. Ardzivian A., Desor F., Soulimani R.

2010	Seminar in Pharmaceutical Biology, Saarland University, Saarbrücken, Germany
	• Evaluation of the neurotoxicity induced by the exposure of lactating dams to the ingestion of the ∑6 NonDioxinLike-PolyChloroBiphenyls in newborn Swiss mice.

2012 France	Seminar in Agence nationale de la recherche (ANR), Université de Lorraine, Metz,
	• Toxicité neurodéveloppementale et comportementale via une exposition lactationnelle à une mixture des 6 PCB-NDL indicateurs chez la souris.
	Séminaire Annuel RP2E 19 janvier 2012, Nancy, France
	• Evaluation des effets neurotoxiques induits chez la souris Swiss suite à l'exposition de la mère allaitante au mélange des 6 PCB-NDL.
	Seminar in Centre de Recherche Public Gabriel Lippmann, Belvaux, Luxembourg
	• Neurotoxicity induced by lactational exposure to six indicator non-dioxin-like polychlorobiphenyls in offspring mice.

Publication

Arpiné Ardzivian Elnar, Britta Diesel, Frédéric Desor, Cyril Feidt, Jaouad Bouayed, Alexandra K. Kiemer, Rachid Soulimani. Neurodevelopmental and behavioral toxicity via lactational exposure to the sum of six indicator non-dioxin-like-polychlorinated biphenyls (∑6 NDL-PCBs) in mice. Toxicology, (2012), 299, 44–54.

Publication in progress

Arpiné Ardzivian Elnar, Christophe Nemos, Frédéric Desor, Sylvain Legay, Torsten Bohn, Rachid Soulimani. Effects of lactational exposure to the sum of six indicator non-dioxin-like-polychlorinated biphenyls (∑6 NDL-PCBs) on oxidative status and mRNA gene expression profiles in mice.

Arpiné Ardzivian Elnar, Ahmad Allouche, Frédéric Desor, Thierry Oster, Rachid Soulimani. Neurotoxicity induced by amyloïd beta-peptide in adult mice lactationnally exposed to the sum of six indicator non-dioxin-like-polychlorinated biphenyls (∑6 NDL-PCBs) at low levels: implications for neurodegeneration in Alzheimer's disease.