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Maternal exposure to a human based mixture of persistent organic pollutants (POPs) affect gene expression related to brain function in mice offspring hippocampus

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HIGHLIGHTS

- POPs detected in the dams were found in offspring brains showing placental transfer.
- POPs exposure led to hippocampal gene expression changes related to brain function.
- External stress combined with POPs caused behavioural deficits in mice offspring.
- The human based POP mixture proved to be useful in prenatal and lactational studies.

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ABSTRACT

Male and female mice pups were exposed to a low and high dose of a human relevant mixture of persistent organic pollutants (POPs) during pregnancy and lactation. Most compounds detected in the dams were found in offspring brains. The mice offspring exhibited changed expression of hippocampal genes involved in cognitive function (*Adora2a*, *Auts2*, *Crlf1*, *Chrnb2*, *Gdnf*, *Gnal*, *Kcnh3*), neuro-inflammation (*Cd47*, *Il1a*), circadian rhythm (*Per1*, *Clock*), redox signalling (*Hmox2*) and aryl hydrocarbon receptor activation (*Cyp1b1*). A few genes were differentially expressed in males versus females. Mostly, similar patterns of gene expression changes were observed between the low and high dose groups. Effects on learning and memory function measured in the Barnes maze (not moving, escape latency) were found in the high dose group when combined with moderate stress exposure (air flow from a fan). Mediation analysis indicated adaptation to the effects of exposure since gene expression compensated for learning disabilities (escape latency, walking distance and time spent not moving in the maze). Additionally, random forest analysis indicated that *Kcnh3*, *Gnal*, and *Crlf1* were the most important genes for escape latency, while *Hip1*, *Gnal* and the low exposure level were the most important explanatory factors for passive behaviour (not moving). Altogether, this study showed transfer of POPs to the

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offspring brains after maternal exposure, modulating the expression level of genes involved in brain function.

1. Introduction

Halogenated persistent organic pollutants (POPs) are of concern to human health and wildlife because of their potential toxicity. Many are structurally closely related and share common characteristics, most notably their persistence and ubiquitous distribution in the environment, their potential for bioaccumulation in living organisms and for bio-magnification in the food chain (de Wit, 2002; Carpenter, 2006; Yogui and Sericano, 2009; Butt et al., 2010; Letcher et al., 2010; Salamova and Hites, 2011). This has led to POPs being banned from production via the Stockholm Convention or regulated through REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), however, a huge number of chemicals that we are exposed to in our daily life are not even tested for toxic properties. Due to this substantial number of compounds and their ubiquitous nature within the environment, cocktails of POPs are typically found in both humans and animals. For example, human umbilical cord blood has been reported to contain more than 200 xenobiotics. Halogenated POPs are present in the blood of children, as well as in breast milk (Aarem et al., 2016; Caspersen et al., 2016), they pass the blood-brain barrier (BBB) (Seelbach et al., 2010; Zhao et al., 2016; Wang et al., 2018) and accumulate in brain tissues (Jones and de Voogt, 1999; Olsen et al., 2007). The infant's body burden of POPs increases with length of breastfeeding, sometimes to levels higher than those of the mother. The prenatal period is a sensitive life stage for adverse effects of toxicant exposure on cognitive development, as brain development at this stage involves processes like neural tube formation and brain segmentation, proliferation and differentiation of different progenitor cell types, apoptosis, migration, phenotypic specification of neurons, and formation of receptors (Stiles and Jernigan, 2010; Kang et al., 2011; Smirnova et al., 2014; Bal-Price et al., 2018). These events create a different window of vulnerability to toxicant exposure (Rice and Barone, 2000; Smirnova et al., 2014). Thus, there is a need for understanding how environmental toxicants in neonates can affect an individual's health and development. The traditional approach has been to study single compounds at relatively high doses, but subsequent research would indicate that toxicants can have biphasic response curves, and/or additive, synergistic, or antagonistic effects on biological endpoints (Altenburger et al., 2013; Bopp et al., 2018). Therefore, effects can be difficult to predict from modelling the results of individual compounds. Consequently, there is a need to focus on the effects of human relevant mixtures.

There is a growing concern that many chemicals present in the environment are potential developmental neurotoxicants (Fritsche et al., 2018). Epidemiological literature has documented associations between neurodevelopmental disorders (Thapar et al., 2017) and prenatal and/or postnatal exposure to neurotoxicants, including lead, mercury, air pollution particulate matter ($PM_{2.5}$), pesticides and flame retardants (Jacobson and Jacobson, 1997; Bjorling-Poulsen et al., 2008; Eskenazi et al., 2008; Costa et al., 2017; Myhre et al., 2018b). However, despite decades of research, epidemiological studies have only enabled to establish associative relationships between POPs and neurodevelopmental effects. Controlled animal experiments provide a possibility to achieve mechanistic support to observational studies. There is evidence that low-level exposures during critical periods of brain development, which would have limited adverse effects in adults, can cause permanent disruptions of normal maturational processes (Rice and Barone, 2000; Grandjean and Landrigan, 2006). However, a critical data gap in human hazard information exists for several classes of POPs despite the fact that they have been commercialized for years (Lindstrom et al., 2011; Linares et al., 2015; LaKind et al., 2018; Lehmann et al., 2018). Review papers largely confirm the difficulty of appraising the body of evidence for a given neurodevelopmental or neurobehavioural outcome after exposure to mixtures of toxicants (Zhang et al., 2017; Harris et al., 2018). Collectively, when considering general effects that may be attributed to chlorinated, brominated or perfluorinated toxicants, studies suggest both pathological and sub-clinical neurodevelopmental and neurobehavioural effects. However, inconsistencies appear concerning health outcomes linked to specific toxicants (Zhang et al., 2017; Harris et al., 2018).

In humans, disorders of neurobehavioural development affect 10–15% of all births. The prevalence rates of such disorders are increasing (Bloom et al., 2010), and several reports express concerns that hazardous substances in the environment may be among the contributing causes of adverse neurodevelopment (Mendola et al., 2002; Alexander et al., 2011; Liu and Schelar, 2012; Grandjean and Landrigan, 2014; Martin et al., 2017; Tung et al., 2017). Subclinical decrements in brain function are even more common than diagnosed disorders, and can have severe consequences for quality of life, reduce academic achievement, disturb behaviour, in addition to being a huge economic burden for society (Grandjean and Landrigan, 2014; Bellanger et al., 2015).

Previously, we designed an environmentally relevant mixture of POPs for use in animal and *in vitro* experimental studies, containing 29 different chlorinated, brominated, and perfluorinated compounds (Berntsen et al., 2017). The *in vivo* POP mixture was composed based on human estimated daily intake (EDI) in Scandinavia, and aimed to provide a realistic mixture for toxicity studies based upon the relative levels of POPs to which individuals are exposed.

In mice used in the present study we recently reported that foetal and lactational exposure to the defined POP mixture affected testicular development, sperm production and sperm chromatin integrity (Khezri et al., 2017b). Additionally, in the same experiment, we found a subtle dysregulation of the hypothalamus-pituitary-adrenal axis (Hudecova et al., 2018). Recent *in vitro* studies with the POP mixture suggest adverse effects on neuronal cell function and development (Berntsen et al., 2021; Davidsen et al., 2021). In the current study, we explore whether the human based POP mixture affects the expression of hippocampal genes relevant to neuronal function and development in mouse offspring and to which extent such changes are reflected in behaviour and cognition. To support biological plausibility, we measured levels of the POPs in maternal and offspring blood and offspring brain tissue.

2. Materials and methods

2.1. Ethics statement

The study was performed at the Section for Experimental

Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The unit was licensed by the Norwegian Animal Research Authority (NARA) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC; FOTS: 5583) and NARA (2013/39783).

2.2. Chemicals and feed

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). In brief, all polybrominated diphenyl ethers (PBDEs), PCBs and other organochlorines used for exposure were originally purchased from Chiron AS (Trondheim, Norway). All perfluorinated compounds (PFASs) and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey) intended for human consumption. All solvents were thoroughly evaporated under N_2-flow before the oil containing the POPs was sent to the feed company (TestDiets, St.Louis, MO) to be incorporated in the mouse feed. Four different diets were made, consisting of three exposure diets for pregnant mice (i.e. control (non-exposed), low dose (5000 × EDI) and high dose (100000 × EDI), and a reference diet for males and pups after weaning. For the control feed, solvents were added to the corn oil at identical levels to those found in the two exposure diets, whereas for the reference feed only untreated corn oil was used. In all diets, the soybean oil in the original feed recipe was exchanged with corn oil intended for human consumption. This was done to reduce background POP exposure, and to limit the amount of phytoestrogens, suspected to interfere with hormone homeostasis, and known to be present in soy-based food (Berntsen et al., 2017).

2.3. Animals, housing, exposure and necropsy

The animals were housed in open type III cages (Tecniplast, Buguggiate, Italy) on standard aspen bedding (Scanbur BK, Nittedal, Norway) and had cellulose nesting material. The animals were fed *ad libitum* and had access to tap water in standard drinking bottles (Tecniplast, Buguggiate, Italy) at all times. The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C with 20 air changes per hour and 45 ± 5% relative humidity.

Altogether, 47 female hybrid mice (129:C57BL/6F1) produced in our facility by 10 male C57BL/6J mice and 20 female 129S1/SvImJ (Jackson Laboratory, Maine, USA), were randomly assigned to three exposure groups, control (n = 15), low dose POP-mixture exposure (n = 16) and high-dose POP-mixture exposure (n = 16). Exposure started at weaning and lasted six months until this generation of dams was euthanized and necropsied. Four weeks after start of exposure, the female mice were mated with a non-litter mate, random hybrid male from the same generation. Two females were housed with one male for one week. One week before expected delivery, the females were single housed until weaning of their pups. After weaning at 3 weeks of age, the offspring were separated from the dams prior to testing in the BM. Power analysis based on a pilot study suggested a group size of 36 offspring (18 males and 18 females from 15 or 16 litters in each exposure group) in the BM test setup. We used randomization to decide which offspring in each litter should be used in the BM tests. These mice were then housed in non-sibling pairs with mice allocated for a different behaviour test (published in (Hudecova et al., (2018)). This randomization eliminates the effect the parents may have had on the litter and the effect of being in a specific cage may have had on the individual. The offspring were given a feed without added POP mixtures. As a result, these offspring were only exposed during foetal life and the three first weeks post-partum through milk and directly from the dam's feed when starting to nibble solid food. After completion of behavioural experiments, tissue and blood for the chemical analyses were obtained through necropsy at 9–10 weeks of age. Thus, the offspring were necropsied 6–7 weeks after end of exposure, while dams were exposed all the way through to necropsy. Body weights were not affected by exposure in either offspring or dams (Hudecova et al., 2018).

At necropsy, the mice were terminally bled from the heart under general anaesthesia (Isoflurane gas 4.5% ISO at 700 mL airflow, followed by 2.5% ISO at 100 mL airflow). Blood plasma was stored at −80 °C until pooling and analyses. The mice were decapitated and the brain gently removed from the skull and divided into two halves. The right brain half was frozen intact, while from the left half, the remaining parts after removal of hippocampus and some other regions of interest (olfactory bulb, prefrontal cortex, pituitary, cerebellum and pons), were stored for chemical analyses. Retroperitoneal adipose tissue adjacent to left kidney and dissected brain parts were frozen on dry ice and stored at −80 °C.

2.4. Behavioural testing in the Barnes maze

The Barnes maze is a commonly used test of learning and memory in rodents (Barnes, 1979). The test was set up in the animal room next to the housing room to reduce the transport time and change of environment. F1 pups exposed to POPs, in addition to control animals, were tested at 10–12 weeks of age. The horizontally placed Barnes maze table consisted of a non-reflective grey circular disk (100 cm in diameter, placed 60 cm above the floor; purchased from Noldus Information Technology, Wageningen, the Netherlands) with 20 holes (5 cm in diameter) located around the perimeter 2.5 cm from the edge. A black, stainless steel escape box (6.4 cm × 20.3 cm x 3.8 cm; equipped with an easy, accessible entrance tunnel made of steel mesh) was magnetically attached beneath one predefined hole (drawn by lottery) and its location remained constant for each mouse for the duration of the study. A 300 W halogen lamp was used as a motivator for the animals to find the escape box (all sessions), positioned above the maze to provide an aversive bright light stimulus (approximately 900 lx at the centre of the table, and 520–550 lx at the edges of the table). To further increase the motivation of the animals a fan blew air onto the maze on day three (session 5). Visual cues (black and white patterns) in plain sight of the animal were located on three of the walls surrounding the maze. An overhead video camera and Ethovision XT 11.0 tracking software (Noldus) were used to record and measure the behaviour of the animals. The animal was placed inside a transparent cylinder (Tecniplast rat play tunnel from Scanbur BK Nittedal, Norway) in the centre of the arena and left there for 30 s. When released from the cylinder, each mouse was tracked until it reached the goal box or for 4 min. Had the animal not located the goal box by 4 min, the mouse was gently guided to the box. The maze, the cylinder and the escape box were carefully cleaned using water and allowed to dry between each trial. The maze was turned 90° clockwise between each session, to avoid fragrance confounding between each session. The testing was done in the animal's light cycle, between 09.00 a.m. and 04.00 p.m. on three consecutive days, two sessions per day (morning and afternoon separated by 4 h). Endpoints studied were: time not moving (seconds), time spent in entry zone (seconds spent in the zone around the escape whole) without entering the escape box, primary latency (seconds until the nose tip touched the edge of the escape box whole), escape latency (seconds from the animal was

released at the centre of the table to entering the escape box), and distance travelled before entering the escape box (meters). All deviations were recorded continuously during testing. Male mice were tested before females. After each session, the noise intensity was recorded. The noise was measured in decibel and recorded with a Castle GA 112 Sound Level Meter (Presisjons Teknikk AS, Oslo, Norway) and background noise was stable around 32 dB.

2.5. Chemical analysis

The measurements were performed at the Norwegian University of Life Sciences (NMBU), Department of Food Safety and Infection Biology, Laboratory of Environmental Toxicology. In order to obtain enough plasma and tissue, and to reduce expenses, one pool of plasma from each exposure group (including both sexes of offspring) and tissue was made prior to chemical analyses. Plasma pools were made by pipetting 150 μl or 50 μl from each dam and offspring sample, respectively; except for from those few animals from which no or a very small sample had been obtained. The whole remaining brain part, kept for chemical analyses, were used for the pools, whereas the adipose tissue was cut with a scalpel blade while kept frozen at −10 °C in a cryostat and one half of it pooled according to exposure group.

The levels of PCBs, OCPs, brominated and PFASs were all measured in plasma and brain tissue, while only the lipophilic PCBs, OCPs and brominated compounds were also measured in adipose tissue. PFASs mainly bind to proteins and are not expected to accumulate in fat (Chen and Guo, 2009).

For the lipophilic group, extraction with cyclohexane/acetone and water was followed by gel permeation column or sulphuric acid clean-up. Separation and detection of the OCPs and PCBs were performed on a GC coupled to Electron Capture Detector (ECD) and low resolution mass spectrometry (LRMS). Detection of BDEs and HBCD was performed on a HRGC−LRMS. Plasma samples analysed for OH-metabolites were extracted as the other plasma samples, but with 1 M H_2SO_4 instead of water. The organic phases from this extraction were analysed by GC-MS like the other plasma samples. For perfluorinated compounds, the samples were extracted with methanol and clean up was accomplished using active carbon. Further, the samples were separated by high-performance liquid chromatography (HPLC), and detection achieved by tandem mass spectrometry (MS-MS). Details from the extraction, clean-up and instrument run for the samples and quality control parameters can be found in Supplementary Information.

The mixture reflected the levels of POPs found in a Scandinavian food basket. A literature review identified the most relevant POPs and the EDI levels of these compounds for a human of 70 kg. Based on the human EDI, corresponding EDIs of the different compounds for a 25 g mouse were calculated. Due to the possibility of background exposure via the mice feed and interspecies differences in compound metabolism, the feed concentration of the mixture was set to provide a mouse consuming 3g feed/day a daily dose of 5000 × and 100000 × the EDI for humans. Following exposure, the level of POPs within the feed (Berntsen et al., 2017) and various tissues (blood plasma, adipose tissue, brain) of the mothers and pups was determined. A list of the individual compounds in the mixture and their measured feed concentrations is shown in the Supplementary Table S1 for further information.

2.6. Gene transcription analysis

Quantitative real-time PCR (qPCR) was used to analyse the transcriptional responses in pooled samples of hippocampus of male and female offspring maternally exposed to the two POP mixture concentrations (low dose (n = 34), and high dose (n = 33)). We investigated a panel of 142 genes by qPCR. Samples from exposed animals were compared to the controls. From the results of initial screening, genes with expression level difference more than ± 2-fold or $p < 0.05$ between POP-mixture exposed and control mice were selected for single sample analysis. Based on these selection criteria, 20 genes were identified.

Total RNA was isolated from frozen (−80 °C) mouse hippocampi (approximately 14 mg tissue) using a ZR-Duet™ DNA/RNA MiniPrep (Zymo research) according to the manufacturer's instructions. The quantity and quality of isolated RNA was determined as previously described (Aaremet al. 2016; Dualeet al. 2014) using a NanoDrop Spectrophotometer (Thermo Scientific, Norway) and Agilent 2100 Bioanalyzer (Agilent Technologies, Norway). RNA purity was estimated by examining the OD 260/280 and the OD 260/230 ratios. RNA integrity numbers (RIN) from 1 to 10 (low to high RNA quality) were calculated using the 2100 Expert software (Agilent Technologies, Norway).

The cDNA synthesis was carried out (Aarem et al., 2016) with 100 ng total RNA from samples as template, using the miScript II RT kit including 5x miScript HiFlex Buffer (for selective conversion of mRNA into cDNA) according to the manufacturers protocol (Qiagen, Norway). A no reverse transcriptase control (NRT) was included and all cDNA samples were stored at −20 °C prior to gene expression analysis.

In the initial screening, cDNA (1:100 dilution) from each treatment group was divided into four female and four male groups and each group consists of pooled cDNA (3−4 cDNA samples) from each sex (i.e., resulting in eight pooled independent samples (4 female and 4 male samples)/treatment group), and for each pooled cDNA, two technical replicates were run. This qPCR layout allowed simultaneous measurement of all samples in one 384-well plate for each gene, reducing errors due to run-to-run variations. Gene-specific qPCR was carried out as previously described (Gutzkow et al., 2016) using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, London, UK) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Norway). Gene-specific primers were designed using the online Universal Probe Library System (Roche Applied Systems, Oslo, Norway). All PCR reactions were performed in duplicates, and data are expressed as an average of the duplicates. The raw-Cq values were analysed by the comparative Cq−method (Livak and Schmittgen, 2001; Duale et al., 2012, 2014). In brief, the stability of nine reference genes (*Actb*, *Gapdh*, *Hprt1*, *Pgk1*, *Rpl13a*, *Tbp*, *Tubb5, Hspb1* and *Ymhaz*) were evaluated by NormFinder algorithm (Andersen et al., 2004) and the most stably expressed reference genes were used for normalization. The results of the stability of the reference genes are presented in Supplementary Fig. S1. Prior to normalization, the raw data Cq values were pre-processed and outliers were excluded from further analysis. In addition, target genes with Cq values > 37 were considered beyond the limit of detection and excluded from further analysis. In the initial screening, target genes were normalized using the average of five stably expressed reference genes (*Actb*, *Gapdh*, *Rpl13a*, *Tbp* and *Ymhaz*) [this is given by ΔCq; where ΔCq (sample) = Cq (target gene) − Cq (average reference genes)]. The ΔΔCq values were generated by subtracting the ΔCq-value for the reference samples (calibrators; vehicle-treated control samples) from the ΔCq-value for the samples [$\Delta\Delta Cq = \Delta Cq$ (sample) − ΔCq (calibrator); fold change = $2^{-\Delta\Delta Cq}$]. The fold change values were then log2-transformed in order to make the values symmetrical around zero. From the initial screening results, 20 genes were selected and their transcriptional levels were analysed by qPCR in all samples; i.e. 33-34 mice/treatment group × 2 technical replicates. The raw *Cq* values were analysed by the comparative *Cq*-method as described above and the following reference genes (*Actb*, *Gapdh*, *Hprt1*, *Pgk1* and *Tubb5*) were used to normalize the

target genes.

2.7. Statistical analysis

Initially, the gene expression data were subjected to univariate analyses (e.g. one-way ANOVA) to investigate exposure effects (ΔCq of POP-mixture exposed samples versus ΔCq of controls). Normal distribution and equality of variances were tested for all data. For logarithm of each of the five behavioural outcomes, namely, time not moving (in seconds), time spent in entry zone (in seconds), primary latency (in seconds), escape latency (in seconds) and distance travelled (in meters), we analysed the difference between the first and fourth sessions as one outcome and the response at the fifth session as the second outcome. The association between exposure groups and behavioural outcomes were evaluated using a linear regression modelling approach, while adjusting for sex and accounting for interaction between exposure and sex. Effect estimates (along with 95% confidence intervals) reported from the analyses can be interpreted as the average change in outcome in the exposed group (High or Low) compared to the unexposed group.

In order to test whether genes are associated with exposure, we investigated the association of gene expression levels of the 20 significantly identified genes from the initial screening with exposure using linear regression models, while adjusting for sex and interaction between exposure levels and sex. Given that some genes are highly correlated with each other, we carried out a principal component analysis (PCA) and computed principal component scores that captured the variability of the gene expression. We considered the first principal component as the mediator in a causal mediation analysis to test whether the effect of exposure on behavioural outcomes were mediated through gene expression (Imai et al., 2010). Each mediation analysis was repeated for low and high exposure levels compared to the unexposed group. Average causal mediated effects, direct effects and total effects are reported for each outcome (difference between the first and fourth sessions as one outcome and the response at the fifth session as the second outcome). The implementation of Random Forests (RF) analysis on two behaviour outcomes (Log escape latency and Log time not moving) provided us with the relative importance of each gene and the exposure levels in predicting the behaviour endpoints. The RF algorithm has become a commonly used machine learning algorithm for genetic association studies, since it is a tree based model that can model interactions between multiple genes well (Goldstein et al., 2011).

Statistical analyses were carried out in R statistical software (Version 3.6.0., R Development Core Team, http://www.r-project.org) (Tingley et al., 2014). P-values < 0.05 were considered statistically significant. The R scripts can be found in the Supplementary Material.

3. Results

3.1. Concentration of chemicals

A dose-dependent increase (low vs high) was observed in both dams and offspring, for all detected compounds in all tested tissues. For exposed groups, the POP levels in dam plasma and tissues were expectedly higher than in offspring. All compounds detected in the dams were also detected in at least one, while most in all, offspring tissue compartments examined. This indicates placental and/or lactational transfer of most of the included compounds (Tables 1 and 2, Supplementary Table S1.

3.2. Concentration of POPs in mouse plasma and adipose tissue

In plasma, the perfluorinated compounds were most abundant on a wet weight basis, PFOA (ng/g) and PFOS (ng/g) dominating in both the dams and offspring generations, respectively. Additionally, PFDA was also found at high concentrations (ng/g) in all groups. For the chlorinated group of compounds, the highly chlorinated PCBs 138 and 153, as well as dieldrin dominated in all groups. Oxychlordane and PCB 180 were further among the most abundant compounds in the offspring, whereas *p,p*'-DDE was prominent in the dams (Table 2). For the brominated compounds, BDE 209, its breakdown product BDE 207 and BDE 47 dominated in the dams, whereas only a few congeners could be detected in the offspring (BDE 100 and 207, and BDE 100, 153 and 154 in the low and high groups, respectively). In adipose tissue, chemicals from the chlorinated group were observed to be the most prominent, with many of the same compounds as for blood dominating, PCB 153 and PCB 138 being the most abundant in all exposure groups (Supplementary Table S1).

3.3. Concentrations and accumulation of POPs in brain

Most compounds the dams were exposed to, were also detected in offspring brain samples. Highly chlorinated PCBs were dominating in brains from mothers and offspring followed by HCB and dieldrin (Table 1). Among the brominated compounds, BDEs 47, 99 and 100 were the most prominent congeners in the dam brains, while the higher brominated BDE 209 and 207 were the predominant in the offspring brains. PFOS, PFUnDA and PFDA were the most abundant PFAS congeners in brains from all exposure groups.

Brain/plasma ratios of measured compounds are shown in Supplementary Tables S2A and S2B. The compounds which were most efficiently transferred from blood to brain were HCB in all low exposure groups, α-HCH in dams, β-HCH in both generations, BDE 209 of the low dose group of dams and BDE 209 in offspring (Supplementary Table S2A). α-HCH shows a high brain/blood-ratio in low exposed dams, while in high exposed dams it was only detected in brain and not in blood. Further, it was not detected in brain or blood in offspring. β-HCH was found at higher levels in the brain than in blood of the low exposed offspring. BDE 209 was absent from offspring blood, but present at almost equal amounts in brain and fat when lipid adjusted, with a brain/adipose tissue ratio of 0.9 (Supplementary Table S2A). The best transferring PFAS from blood to brain was PFUnDA, with a much higher fraction of the blood levels being deposited in the brain than for any of the other PFAAs (Supplementary Table S2B).

3.4. Metabolites in plasma

Eleven chlorinated and five brominated hydroxy-metabolites were analysed in plasma from control and high exposed dams of which six chlorinated were detected in the high exposed dams (Supplementary Table S3**)**.

3.5. Human relevance

To predict the relevance of the current exposure scenario and POP mixture for human health outcomes, we compared levels in mice plasma to the average blood levels (ng/ml) of POPs from the Scandinavian population, published in (Berntsen et al., (2017)). This comparison reveals that for some compounds, plasma levels in the low dose offspring group are human relevant. *p,p'*-DDE in humans is in the same range and even higher than the levels measured in this group. Further, most of the PCB congeners are only 10 times higher than in humans. The BDEs on the other hand, were mostly

Table 1
Levels of POPs in brains (ng/g wet weight) in pooled samples from dams and pups of the different exposure groups and generations in mice exposed to a human relevant POP mixture. The values for each group of compounds (range) are listed from the highest to lowest measured concentration. The three most prominent compounds within each exposure and compound group are highlighted in a dark grey colour.

Range	Dam						Offspring					
	Control		Low		High		Control		Low		High	
Chlorinated												
1	HCB	1.12	PCB 138	33.22	PCB 138	646.16	HCB	0.69	PCB 153	6.78	PCB 153	140.2
2	PCB 153	0.36	HCB	31.22	PCB 153	635.38	PCB 138	0.06	PCB 138	6.68	PCB 138	128.35
3	PCB 180	0.09	PCB 153	28.79	HCB	535.07	PCB 118	0.03	Dieldrin	5.51	Dieldrin	54.59
4	PCB 28	n.d.	Dieldrin	27.26	Dieldrin	344.52	PCB 28	n.d.	HCB	4.99	HCB	54.2
5	PCB 52	n.d.	*p,p′*-DDE	18.87	PCB 118	303.97	PCB 52	n.d.	β-HCH	3.22	PCB 118	38.58
6	PCB 101	n.d.	β-HCH	15.02	PCB 180	184.48	PCB 101	n.d.	PCB 180	1.81	PCB 180	35.13
7	PCB 118	n.d.	PCB 118	10.96	*p,p′*-DDE	155.21	PCB 153	n.d.	Oxychlordane	1.47	Oxychlordane	32.02
8	PCB 138	n.d.	PCB 180	8.67	Oxychlordane	125.89	PCB 180	n.d.	PCB 118	1.14	β-HCH	23.88
9	*p,p′*-DDE	n.d.	Oxychlordane	7.28	β-HCH	80.41	*p,p′*-DDE	n.d.	*trans*-Nonachlor	0.88	PCB 52	16.4
10	α-Chlordane	n.d.	*trans*-Nonachlor	5.35	*trans*-Nonachlor	65.07	α-Chlordane	n.d.	*p,p′*-DDE	0.69	*trans*-Nonachlor	13.12
11	Oxychlordane	n.d.	α-HCH	4.07	α-HCH	22.33	Oxychlordane	n.d.	PCB 28	n.d.	*p,p′*-DDE	3.02
12	*trans*-Nonachlor	n.d.	PCB 101	2.72	PCB 52	8.22	*trans*-Nonachlor	n.d.	PCB 52	n.d.	PCB 28	n.d.
13	α-HCH	n.d.	PCB 52	0.76	PCB 101	7.26	α-HCH	n.d.	PCB 101	n.d.	PCB 101	n.d.
14	β-HCH	n.d.	PCB 28	0.22	PCB 28	2.88	β-HCH	n.d.	α-Chlordane	n.d	α-Chlordane	n.d
15	γ-HCH	n.d.	α-Chlordane	n.d.	α-Chlordane	n.d.	γ-HCH	n.d.	α-HCH	n.d	α-HCH	n.d
16	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.	Dieldrin	n.d.	γ-HCH	n.d	γ-HCH	n.d
Brominated												
1	BDE 47	n.d.	BDE 47	3.7	BDE 47	56.44	BDE 47	n.d.	BDE 209	0.96	BDE 207*	4.99
2	BDE 99	n.d.	BDE 209	2.11	BDE 99	21.51	BDE 99	n.d.	BDE 100	0.4	BDE 209	3.67
3	BDE 100	n.d.	BDE 100	1.72	BDE 100	20.27	BDE 100	n.d.	BDE 207*	0.4	BDE 153	3.28
4	BDE 153	n.d.	BDE 99	1.7	BDE 209	17.95	BDE 153	n.d.	BDE 99	0.18	BDE 100	1.44
5	BDE 154	n.d.	BDE 207*	1.35	BDE 153	17.67	BDE 154	n.d.	BDE 153	0.15	BDE 99	1.18
6	BDE 196*	n.d.	BDE 153	0.83	BDE 207*	12.88	BDE 196*	n.d.	BDE 154	0.07	BDE 154	0.39
7	BDE 202*	n.d.	BDE 154	0.57	BDE 154	6.58	BDE 202*	n.d.	BDE 47	n.d.	BDE 47	n.d.
8	BDE 206*	n.d.	BDE 196*	n.d.	BDE 208*	2.47	BDE 206*	n.d.	BDE 196*	n.d.	BDE 196*	n.d.
9	BDE 207*	n.d.	BDE 202*	n.d.	BDE 196*	n.d.	BDE 207*	n.d.	BDE 202*	n.d.	BDE 202*	n.d.
10	BDE 208*	n.d.	BDE 206*	n.d.	BDE 202*	n.d.	BDE 208*	n.d.	BDE 206*	n.d.	BDE 206*	n.d.
11	BDE 209	n.d.	BDE 208*	n.d.	BDE 206*	n.d.	BDE 209	n.d.	BDE 208*	n.d.	BDE 208*	n.d.
12	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.
Perfluorinated												
1	PFHxS	n.d.	PFOS	18.5	PFOS	266	PFHxS	n.d.	PFOS	2.12	PFOS	34.3
2	PFOS	n.d.	PFUnDA	14.4	PFUnDA	139	PFOS	n.d.	PFDA	1.21	PFDA	20.2
3	PFOA	n.d.	PFDA	13.8	PFDA	136	PFOA	n.d.	PFUnDA	1.09	PFUnDA	18.1
4	PFNA	n.d.	PFOA	4.86	PFOA	77.7	PFNA	n.d.	PFNA	0.37	PFNA	6.8
5	PFDA	n.d.	PFNA	3.44	PFNA	42	PFDA	n.d.	PFOA	0.3	PFOA	6.07
6	PFUnDA	n.d.	PFHxS	2.07	PFHxS	35	PFUnDA	n.d.	PFHxS	n.d	PFHxS	2.63
7	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d
8	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d

n.d = Not detected, *= Not added to mixture

Table 2
Plasma levels of POPs (ng/g wet weight) in pooled samples for the different exposure groups and generations of mice exposed to a human relevant POP mixture. The values for each group of compounds (range) are listed from the highest to lowest measured concentration. Human blood wet weight levels (ng/ml) gained from the corresponding *in vitro* mixture, based on the Scandinavian population (Berntsen et al., 2017), are also given for comparison. The three most prominent compounds within each exposure and compound group are highlighted in a dark grey colour.

Range	Human		Dam						Offspring					
			Control		Low		High		Control		Low		High	
Chlorinated														
1	*p,p'*-DDE	0.502	PCB 153	0.126	Dieldrin	12.884	PCB 138	90.814	PCB 153	1.160	PCB 138	3.984	PCB 138	49.756
2	PCB 153	0.362	HCB	0.088	PCB 138	12.032	PCB 153	87.31	PCB 138	1.158	PCB 153	2.891	PCB 153	48.939
3	PCB 138	0.222	PCB 138	0.079	PCB 153	7.435	*p,p'*-DDE	51.444	PCB 180	0.330	Dieldrin	1.355	Dieldrin	16.829
4	PCB 180	0.194	PCB 118	0.070	*p,p'*-DDE	5.337	Dieldrin	50.394	PCB 118	0.282	Oxychlordane	1.285	PCB 180	14
5	HCB	0.117	PCB 180	0.033	HCB	2.874	PCB 118	42.126	HCB	0.274	PCB 180	0.774	Oxychlordane	13.659
6	PCB 118	0.064	PCB 28	n.d.	PCB 180	2.723	HCB	40.551	PCB 28	n.d.	HCB	0.747	PCB 118	11.829
7	β-HCH	0.053	PCB 52	n.d.	PCB 118	2.474	PCB 180	24.4	PCB 52	n.d.	PCB 118	0.369	β-HCH	10
8	*trans*-Nonachlor	0.041	PCB 101	n.d.	Oxychlordane	1.579	Oxychlordane	21.522	PCB 101	n.d.	*trans*-Nonachlor	0.359	HCB	9.512
9	Dieldrin	0.024	*p,p'*-DDE	n.d.	β-HCH	1.547	β-HCH	14.436	*p,p'*-DDE	n.d.	*p,p'*-DDE	0.299	*trans*-Nonachlor	6.22
10	Oxychlordane	0.022	α-Chlordane	n.d.	PCB 101	1.179	trans-Nonachlor	9.843	α-Chlordane	n.d.	β-HCH	0.149	PCB 52	2.805
11	PCB 28	0.013	Oxychlordane	n.d.	PCB 52	1.084	PCB 52	4.724	Oxychlordane	n.d.	PCB 52	0.12	*p,p'*-DDE	1.707
12	α-Chlordane	0.011	*trans*-Nonachlor	n.d.	*trans*-Nonachlor	1.084	PCB 101	2.362	*trans*-Nonachlor	n.d.	PCB 28	n.d.	PCB 28	n.d.
13	PCB 52	0.010	α-HCH	n.d.	α-HCH	0.211	PCB 28	n.d.	α-HCH	n.d.	PCB 101	n.d.	PCB 101	n.d.
14	PCB 101	0.008	β-HCH	n.d.	PCB 28	n.d.	α-Chlordane	n.d.	β-HCH	n.d.	α-Chlordane	n.d.	α-Chlordane	n.d.
15	α-HCH	0.006	γ-HCH	n.d.	α-Chlordane	n.d.	α-HCH	n.d.	γ-HCH	n.d.	α-HCH	n.d.	α-HCH	n.d.
16	γ-HCH	0.006	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.	Dieldrin	n.d.	γ-HCH	n.d.	γ-HCH	n.d.
Brominated														
1	HBCD	0.025	BDE 47	n.d.	BDE 209	4.884	BDE 209	26.115	BDE 47	n.d.	BDE 100	0.09	BDE 154	1.951
2	BDE 209	0.011	BDE 99	n.d.	BDE 47	1.232	BDE 47	10.761	BDE 99	n.d.	BDE 207*	0.159	BDE 153	1.098
3	BDE 47	0.009	BDE 100	n.d.	BDE 207*	0.642	BDE 207*	4.724	BDE 100	n.d.	BDE 47	n.d.	BDE 100	0.61
4	BDE 153	0.010	BDE 153	n.d.	BDE 100	0.526	BDE 99	3.806	BDE 153	n.d.	BDE 99	n.d.	BDE 47	n.d.
5	BDE 99	0.004	BDE 154	n.d.	BDE 99	0.495	BDE 100	3.806	BDE 154	n.d.	BDE 153	n.d.	BDE 99	n.d.
6	BDE 100	0.002	BDE 196*	n.d.	BDE 154	0.263	BDE 153	2.49	BDE 196*	n.d.	BDE 154	n.d.	BDE 196*	n.d.
7	BDE 154	0.002	BDE 202*	n.d.	BDE 153	0.179	BDE 154	0.919	BDE 202*	n.d.	BDE 196*	n.d.	BDE 202*	n.d.
8	BDE 206*	N/A	BDE 206*	n.d.	BDE 196*	n.d.	BDE 196*	n.d.	BDE 206*	n.d.	BDE 202*	n.d.	BDE 206*	n.d.
9	BDE 207*	N/A	BDE 207*	n.d.	BDE 202*	n.d.	BDE 202*	n.d.	BDE 207*	n.d.	BDE 206*	n.d.	BDE 207*	n.d.
10	BDE 208*	N/A	BDE 208*	n.d.	BDE 206*	n.d.	BDE 206*	n.d.	BDE 208*	n.d.	BDE 208*	n.d.	BDE 208*	n.d.
11	BDE 209	N/A	BDE 209	n.d.	BDE 208*	n.d.	BDE 208*	n.d.	BDE 209	n.d.	BDE 209	n.d.	BDE 209	n.d.
12	HBCD	N/A	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.	HBCD	n.d.
Perfluorinated														
1	PFOS	29.43	PFOS	0.89	PFOA	345	PFOA	6980	PFOS	15.9	PFOS	28.8	PFOS	635
2	PFOA	4.52	PFOA	0.53	PFDA	246	PFOS	3403	PFDA	14.2	PFDA	27.5	PFOA	598
3	PFHxS	3.45	PFNA	0.32	PFOS	223	PFHxS	3381	PFNA	13	PFOA	26.1	PFDA	503
4	PFNA	0.80	PFDA	0.25	PFNA	176	PFDA	2580	PFOA	11.5	PFNA	23.5	PFNA	470
5	PFDA	0.50	PFUnDA	0.16	PFHxS	161	PFNA	2531	PFHxS	6.39	PFHxS	15.5	PFHxS	317
6	PFUnDA	0.56	PFHxS	n.d	PFUnDA	63	PFUnDA	724	PFUnDA	3.15	PFUnDA	6.22	PFUnDA	114
7	PFDoDA*	N/A	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	0.81	PFDoDA*	n.d	PFDoDA*	n.d	PFDoDA*	n.d
8	PFTrDA*	N/A	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d	PFTrDA*	n.d

n.d = Not detected, N/A = Not available, *=Not added to mixture

not detected in the offspring, whereas they were up to 450 times higher than human blood levels in the low dose exposed dams. For the perfluorinated compounds, PFOS levels were similar to human blood levels in the low dose offspring. PFHxS and PFOA were 5–6 times higher and the remaining three PFAAs were 10–60 times higher (Supplementary Table S4).

3.6. Gene analysis

The genes selected from the initial screening are presented in Supplementary Fig. S2. Initial screening identified 20 genes (*Adora2a, Alk, Auts2, Bnip1, Cd47, Chrnb2, Clock, Crlf1, Cyp1b1, Gdnf, Gnal, Hip1, Hmox2, Il1a, Kcnh3, Ier3, Mecp2, Per1, Pnpt1* and *Tnf*) that were analysed in all samples (N = 101 samples; i.e., 33 or 34 mice/treatment group). The relative transcript levels are shown in Fig. 1. Thirteen genes (*Adora2a, Alk, Auts2, Cd47, Chrnb2, Clock, Crlf1, Cyp1b1, Gnal, Hmox2, Il1a, Kcnh3 and Per1*) were identified as significant differentially expressed in POP-mixture exposed groups compared to the control group (Fig. 1A). Initial univariate analysis indicated that the expression levels of eight genes (downregulated genes: *Auts2, Adora2a, Gnal, Per1*, and upregulated genes: *Kcnh3, Cyp1b1, Hmox2, Cd47*) were changed more than 2-fold relative to control samples (Supplementary Table S5). Interestingly, three genes (Auts2, *Kcnh3 and Cyp1b1)* were differentially expressed

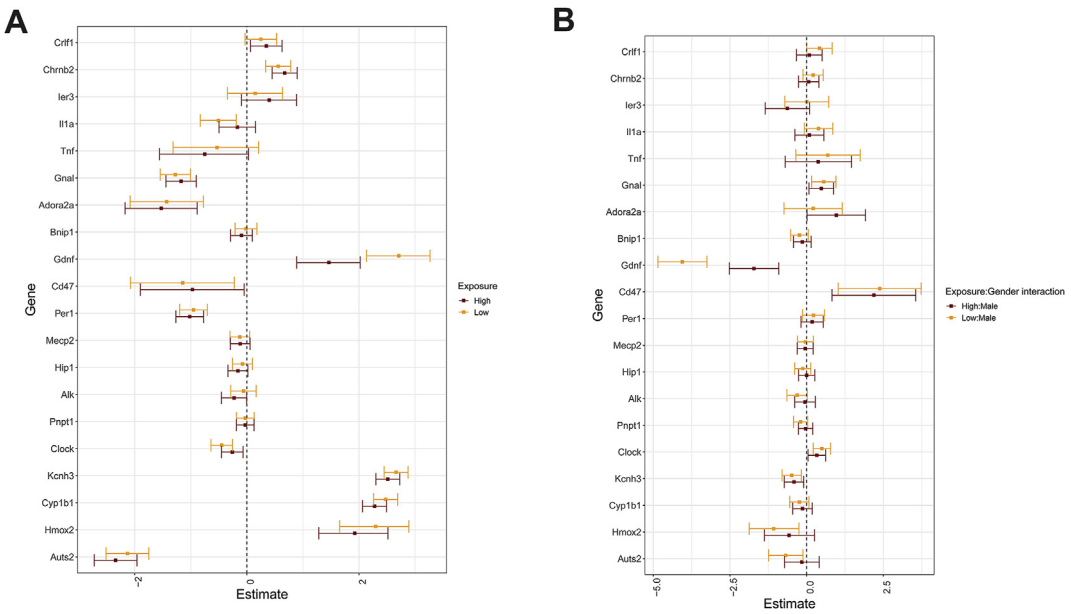

Fig. 1. Average change in gene expression levels in mouse offspring maternally exposed to a human relevant mixture of POPs at two dose levels (high and low) at 10–12 weeks of age, adjusted for sex and interaction between exposure and sex. Fig. 1A illustrates the average change in gene expression between the levels of exposure against the control group. Fig. 1B describes the average change in gene expression between highly exposed males (and low exposed males) compared to females in the control group, representing the interaction effect.

more than 9-fold relative to control samples in both low and high groups. In general, the transcript levels of most of the 20 genes showed similar expression pattern in high and low exposed offspring when compared with controls (Fig. 1). Further, there were no statistical significant differences between genders (males and females) and exposure groups, except for the *Gdnf* gene (Fig. 1). Estimates (fold change) and 95% confidence intervals of association between each gene with main effects of gender and exposure and interaction between gender and exposure under a generalized linear model framework is shown in Supplementary Table S6.

Correlation analysis was conducted with the transcript level of the 20 identified genes. Visual inspection of the correlation heatmap (Supplementary Fig. S3) reveals that some of these genes were highly correlated with each other. Therefore, a PCA was carried out to capture the variability of the gene expression levels.

The first principal component (PC1) of clustered genes explained 36.4% of the variation and was used as a mediator variable in a mediation model. PC1 is dominated by increased and decreased levels of gene expression as shown in the biplot of the components (Supplementary Fig. S4).

3.7. Behavioural testing in offspring

After repeated behaviour testing of the offspring in four consecutive sessions, there was no exposure effect on learning ability, expressed as the difference in behavioural endpoints between session 1 and 4 (Fig. 2A). However, in session 5, when a fan was introduced, offspring of high exposed mothers showed significantly longer time not moving and longer escape latency (Fig. 2B). A similar trend was found for the low dose exposure group, although not statistically significant. Sex did not affect behaviour outcomes.

Mediation analysis indicated that the direct effect of gene transcription levels as expressed by PC1 from PCA contributed with an adaptive response in several behaviour endpoints, including escape latency, distance travelled and time not moving, thereby bringing the total effect towards the normal (e.g. control; Fig. 3). Fig. 4 shows rank ordering of gene expression after random forest analysis, where *Hip1*, *Gnal* and exposure (low dose) were the most important three predictors for not moving, while *Kcnh3*, *Gnal* and *Crlf1* for escape latency.

4. Discussion

4.1. Internal tissue dose levels of the POPs

The toxicants in the POP mixture used in our study were selected for their presence in Scandinavian food products, breast milk or blood, and the mixture was designed based on defined human estimated daily intake levels (Berntsen et al., 2017). The measured concentrations in mouse blood and tissue showed that our exposure regime resulted in concentrations in the low dose group of maternally exposed offspring, comparable to levels in the Scandinavian population. Generally, compounds with high content in the feed were also detected at high concentrations in the tissues, though a few compounds were not detected at all (HBCD, α-chlordane and γ-HCH). The concentration of several POPs in offspring plasma were higher than in the dams (Table 2). All offspring were placed in a separate room after weaning and given the reference feed, which was not added POPs. The reason for the higher levels in control offspring is unknown.

Some compounds showed greater affinity for the brain than other chlorinated (β-HCH, α-HCH, HCB), brominated (BDE 209) and perfluorinated (PFUnDA) compounds.

4.2. Concentration and accumulation of the POPs in brain

The abundance of potential hazardous chemicals in the brains of exposed offspring is of special relevance for brain development and may induce behavioural effects. In this context it is important to emphasize that the mice were terminally bled, and little blood was likely to be left in brain capillaries. It is therefore assumed that the measured concentrations reflect the levels in brain tissues. Although difficult to retrieve for humans, measurements of

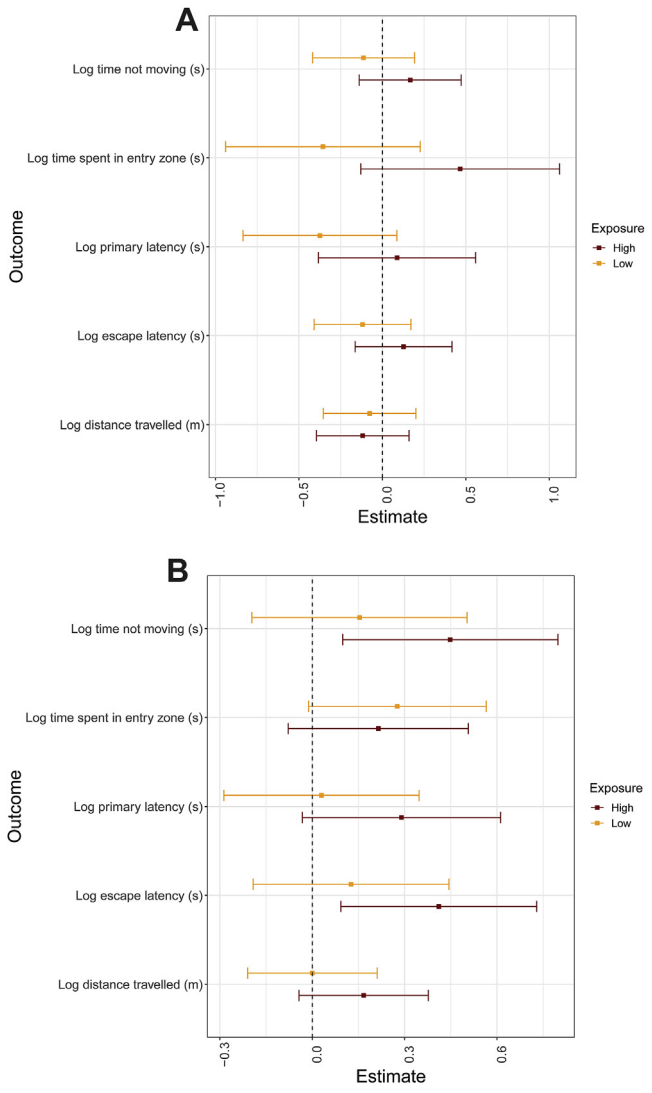

Fig. 2. Average change in behavioural performance in mouse offspring maternally exposed to a human relevant mixture of POPs at two dose levels (high and low) and subjected to the Barnes maze test repeated five times (5 sessions) at 10–12 weeks of age. Fig. 2A illustrates the difference in behavioural outcomes between session 1 and 4 (e.g. the learning effect) and 2B the same outcomes when a noisy fan was introduced to increase the motivation for hiding (session 5).

organochlorine (OC) or PBDE concentrations in human brain tissues (Dewailly et al., 1999) associated to neurological disorders (Corrigan et al., 1996, 1998; Hatcher-Martin et al., 2012; Mitchell et al., 2012) have been performed. OCs included in our experiment occurred in human brains at levels comparable with concentrations in low exposed offspring and low exposed dams in the current experiment, confirming human relevance of the exposure in the present study.

Most compounds detected in dams were also found in offspring brain samples, indicating transfer of these compounds across the placenta as well as the BBB. Small lipid-soluble compounds will diffuse across the BBB, while other compounds would need carrier- or receptor-mediated transport (Goasdoue et al., 2017). Thus, the lipid-soluble POPs such as the chlorinated and brominated compounds will easily cross this barrier, whereas one would expect the less lipophilic, protein binding PFAS to need another transport mechanism.

We observed that the brain/adipose tissue ratio was much higher for BDE 209 than for the other BDEs (Supplementary Table 2A). This may seem contradictory to published data, suggesting highly brominated congeners to penetrate the BBB less efficiently than congeners with lower bromine levels (Zhao et al., 2016). Foetal exposure may lead to higher brain levels of this compound than after postnatal exposure, as shown in rats (Zhang et al., 2011). This seems likely based on the current data where lipid adjusted levels of BDE 209 in brain were almost similar in the dams and offspring of the low exposure group despite a longer and continuous exposure period in the dams. Still, such an effect is difficult to explain as the foetal BBB is functional at an early stage in many mammals, including rodents (Goasdoue et al., 2017).

The fact that PFUnDA transferred best from blood to brain, compared with the other PFAAs (Supplementary Table 2B), could according to Greaves and colleagues be due to higher lipophilicity and brain accumulation of longer chained PFAAs (Greaves et al., 2013). This group also hypothesized that PFASs with different chain length had specific affinity to different proteins and tissues, with certain proteins binding preferentially to PFCAs with a certain chain length (Greaves et al., 2012). Furthermore, the same authors indicated that longer chained PFCAs (C_{10}–C_{15}) may be transported across the BBB via mechanisms resembling the transport of saturated fatty acids (Greaves et al., 2013) which may perhaps explain the increased accumulation of the longer chained PFCAs in the brain. Literature on PFUnDA in the brain is sparse, but it was found to be one of the most abundant PFAS measured in the brains of polar bears from Greenland (Eggers Pedersen et al., 2015). Here, PFUnDA was detected at same average concentrations as PFOS across different brain regions, but at lower levels than PFTrDA (Eggers Pedersen et al., 2015). Also, in brain samples from harbour seals and red-throated divers the longer chained PFAAs such as PFUnDA have been found to accumulate to a higher extent in brain relative to blood, than shorter chained compounds (Ahrens et al., 2009; Rubarth et al., 2011).

4.3. Metabolites of POPs in relation to developmental neurotoxicity

It has been suggested that highly brominated BDEs like BDE 209 may debrominate to more toxic, lower brominated compounds (Martin et al., 2017). Of the five congeners likely to be debromination products and which are marked with an asterisk in Tables 1 and 2 and Supplementary Table S1, only BDE 207 was detected in all measured compartments, whereas BDE 208 was detected less frequently and at lower concentrations. Since brominated congeners detected in the mouse tissues were not present in the feed, debromination has most likely taken place in the animals. BDE 207 is presumably a frequent *in vivo* debromination product of BDE 209, as found in both rats (Wang et al., 2010), cows (Kierkegaard et al., 2007), human (Qu et al., 2007), and in the mouse tissues in the present study.

Hydroxylated (OH) metabolites of the chlorinated and brominated POPs were identified in the current study (Supplemental Table 3), where three out of six OH-metabolites detected in the high exposed dams (4-OH-PCB 107, 3′OH-PCB 138, 4-OH-PCB 146) are among the five most frequently found in human blood (Grimm et al., 2015). The presence of OH-metabolites like 4-OH-CB107 may have toxicological implications since they have shown greater toxicity and increased abundancy compared to the parent compounds (Antunes-Fernandes et al., 2011; Grimm et al., 2015). However, it is an uncommon finding in humans that levels of these metabolites exceed the most persistent and accumulating PCB parent compounds (Grimm et al., 2015). PFASs do not undergo metabolism in the liver or other tissues (Pizzurro et al., 2019), precluding any concern about species extrapolation after

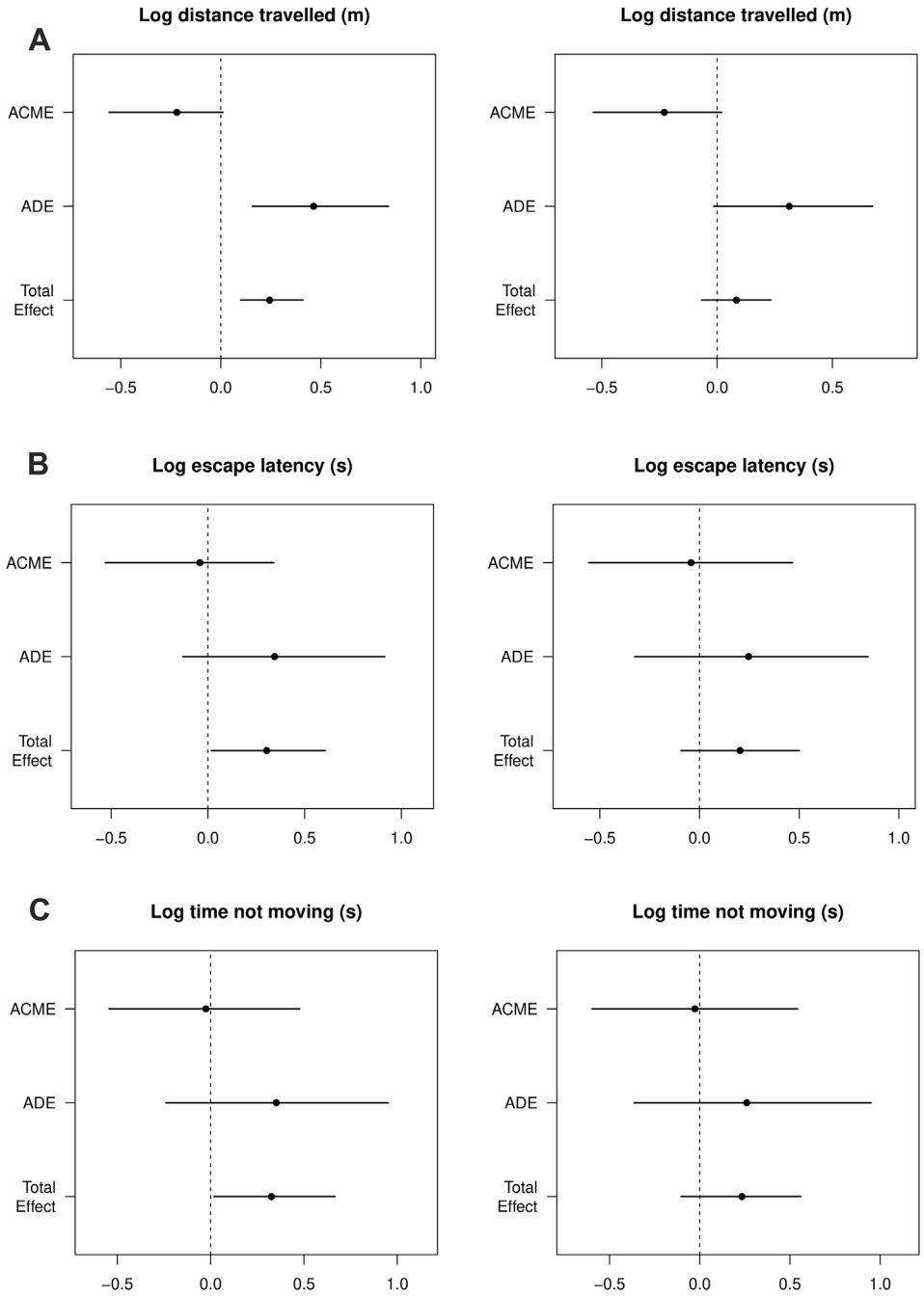

Fig. 3. Estimated average causal mediated effect (ACME), average direct effect (ADE) and total effect of exposure, with mediation by gene transcription levels, on behavioural endpoints in session 5 of the Barnes maze test for high (left panel) and low dose mice (right panel) maternally exposed to a human relevant mixture of POPs. The plots show mean estimates with confidence limits. The zero stippled line is the reference (e.g. control group). Three behavioural endpoints are shown: A) Log transformed escape latency, B) Log transformed distance travelled, C) Log transformed time not moved.

metabolism. Two perfluorinated compounds, perfluorododecanoic acid (PFDoDA) and perfluorotridecanoic acid (PFTrDA), were not added to the feed, but were already in the test panel and therefore measured. Interestingly, they were detected at low levels in the high dose feed (unpublished data) and PFDoDA also in high dose dam plasma (Table 2). As these compounds are longer than the PFAAs added to the mixtures, they cannot be break down products. Rather, they must be contaminants, and as they were only detected in the high dose feed, contamination of the PFAAs used with these longer-chained congeners is the most likely explanation for their presence. The design of our mouse study did not allow determination of the relative importance of metabolites compared to the parent compounds for the observed gene expression changes and behavioural outcomes.

4.4. Hippocampal gene expression levels

An approach to study how cells respond to a stress condition is to investigate how the gene transcription is altered, for instance in relation to disturbed neurodevelopmental and cognitive processes. In exposed mice we observed differential expression of hippocampal genes related to cognitive function with increased

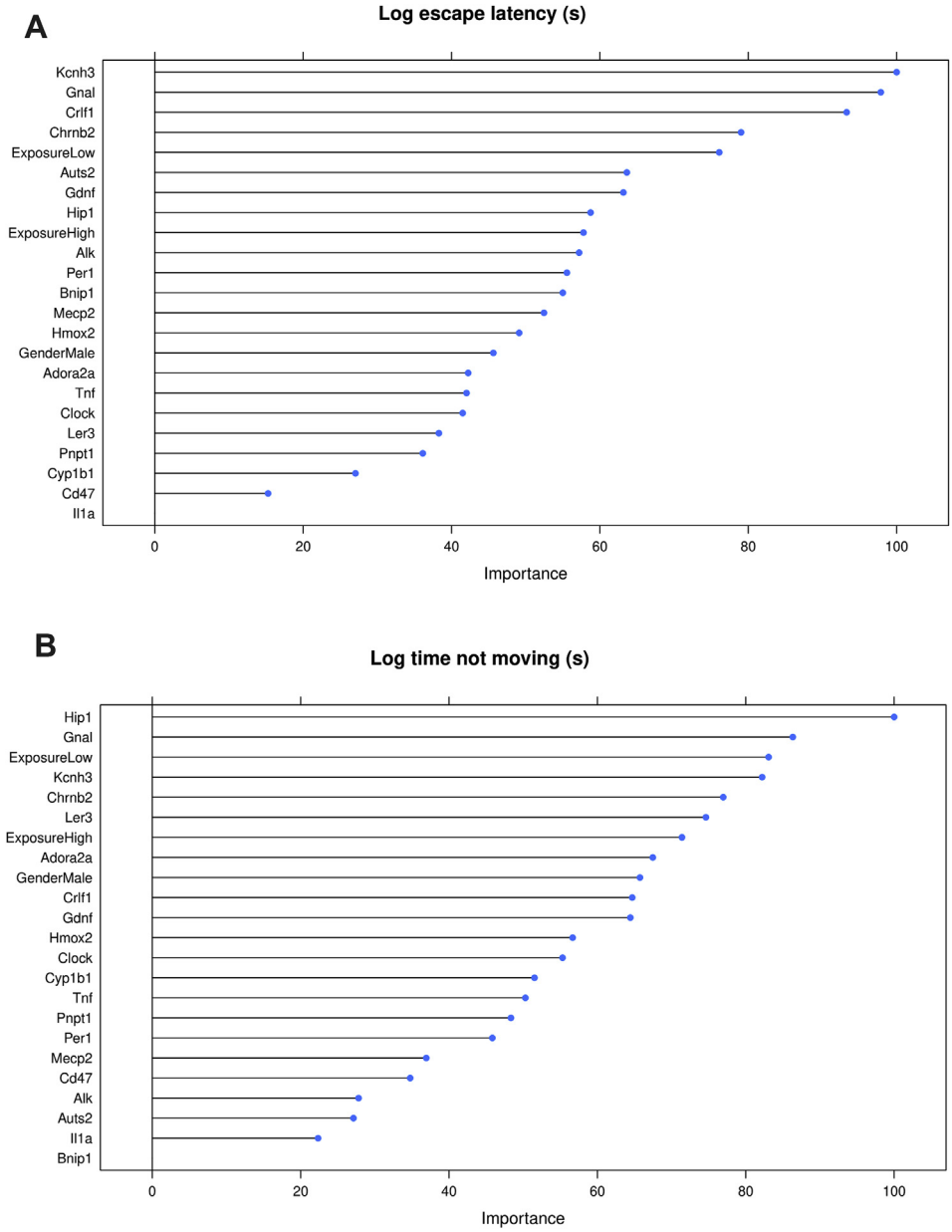

Fig. 4. Random Forest Plots showing the relative importance of gene expression and exposure for behavioural endpoints (log transformed escape latency, Fig. 4A), and the log transformed time spent not moving, Fig. 4B) in session 5 of the Barnes maze test for high and low dose mice maternally exposed to a human relevant mixture of POPs.

expression of *Crlf1*, *Chrnb2*, *Gdnf*, *Kcnh3*, and decreased expression of *Auts2*. Additionally, we found that *Hip1*, *Gnal* and exposure (low dose) were the most important three predictors for not moving, while *Kcnh3*, *Gnal* and *Crlf1* for escape latency. Considering reported neurological function of these genes, the associations between behavioural changes and gene expression adds biological plausibility and mechanistic support to our findings. *Crlf1* encodes a protein that supports differentiation and survival of a wide range of neural cell types during embryonic development and in adult neural tissues (Rousseau et al., 2006). Likewise, in the rodent hippocampal dentate gyrus, the majority of mature and immature granule cells express *Chrnb2* cholinergic receptors (Kaneko et al., 2006), and increased expression of this gene may reflect dysfunctional cholinergic signalling via *Chrnb2* on hippocampal neurogenesis and learning performance. The *Gdnf* gene encodes a neurotrophic factor that contributes to normal hippocampal development (Irala et al., 2016), and hippocampal GDNF protein levels are maintained throughout the lifespan (Werry et al., 2010) suggesting that this protein has a continuous regulatory role of hippocampal function including learning and memory (Cunha et al., 2010).

The *Kcnh3* gene expression level increased significantly in both exposure groups, which is interesting in the context of cognitive function since it is reported that cognitive function is changed when this gene is knocked out (Miyake et al., 2009). It has also been suggested that *Kcnh3* is a potent regulator of excitability in hippocampal pyramidal neurons (Zhang et al., 2010). We further observed a highly significant change in expression level of the *Auts2* gene in exposed animals versus controls. Interestingly, dysregulation of this gene is associated with e.g. autism, mental retardation and developmental delay (Bedogni et al., 2010; Fan et al., 2016). Knockout of both coding and noncoding sequences of the *Auts2*

gene in zebrafish caused microcephaly and a decreased number of neuronal cells (Oksenberg et al., 2013), also seen in ASD patients (Liu et al., 2015).

Gnal encodes a stimulatory G protein alpha subunit which couples dopamine type 1 receptors (D1R) and adenosine A2A receptors (Adora2a). The Adora2a protein has important roles in the regulation of glutamate and dopamine release, and inhibition of this protein has been shown to enhance spatial memory and hippocampal plasticity (Laurent et al., 2016). It has further been reported that *Gnal* ± mice displayed a clear reduction in acute locomotor response to psychostimulant drugs (Corvol et al., 2007). Also, expression levels of e.g. *Gnal* was altered in PCB-exposed rats (DasBanerjee et al., 2008). The expression level of *Gnal* was decreased in both exposure groups compared to control group (Fig. 1).

Hip1 knockout mice show neurological deficits (Metzler et al., 2007), and overexpression of *Hip1* is reported to induce neuronal cell death through apoptosis (Choi et al., 2006). Changes in *Hip1* expression in our study may therefore be a biological attempt to counteract apoptosis.

Altogether, this support that the differentially expressed genes in the exposed animals compared to the controls are likely to affect learning and memory processes in the mouse brain, either directly or as a compensatory mechanism for learning deficits. However, complex processes like cognitive functions are likely to act through networks of genes rather than through single genes.

Our results further show that prenatal exposure to the POP mixture led to dysregulation of genes associated with inflammation, disturbances of circadian rhythm, AhR activation and redox signalling. Interleukins modulate inflammatory responses, and interestingly, several interleukins are linked to cognitive deficits (Misiak et al., 2018). In this respect, negative relationship between *IL-1α* levels in the hippocampus may indicate inflammatory responses and possibly cognitive impairment. CD47 together with SIRPα mediate the interplay between microglia and other brain cells, are important in neuroinflammatory processes and in several CNS disorders (Zhang et al., 2015). Organic compounds can bind the aryl hydrocarbon receptor (AhR), and this may in turn lead to an increased expression of genes linked to inflammation and xenobiotic metabolism (Esser and Rannug, 2015). Our results show that the AhR regulated gene *Cyp1b1* had higher expression in both exposure groups than in the control. Heme oxygenase-1 (*Hmox1*) can be induced in response to toxicants like PCBs (Lee et al., 2006), BFR (Zou et al., 2013), and perfluorinated compounds (Shi and Zhou, 2010), in addition to other stimuli that cause oxidative stress (Keyse and Tyrrell, 1989; Stocker, 1990; Dwyer et al., 1992; Hoshida et al., 1996). Astrocytes and microglia are potent inducers of *Hmox1*. To counter neuroinflammation and oxidative stress, these cells respond by inducing *Hmox1* expression, as shown in our study.

We observed that the clock genes *Per1* (period circadian clock 1) and *Clock* (clock circadian regulator) were downregulated as a response to POP exposure. The proteins encoded by these genes play central roles in the regulation of circadian rhythms. Genes in the Per1 family encode components of the circadian rhythms of locomotor activity, metabolism, and behaviour. *Per1* is upregulated by Clock/Arntl heterodimers. Disruption of several individual clock genes throughout the brain can impair hippocampal long-term memory in young animals, possibly gating memory formation dependent on the time of day (Kwapis et al., 2018).

Estimates of gene expression patterns comparing sex effects revealed higher expression of *Gnal*, *Adora2a*, *Cd47*, *Clock*, and lower expression of *Kcnh3*, *Hmox2*, and *Auts2* in males versus females (Fig. 1B). The biological importance of these differences is challenging to explain based on our mouse study, since we did not reveal any statistically significant differences in cognitive performance between the sexes. However, it could be that these genes affect other neurodevelopmental cognitive outcomes than those measured in the Barnes maze. For example, males are more susceptible to ADHD and autism while females suffer more from mood disorders such as depression and anxiety (Pinares-Garcia et al., 2018; May et al., 2019). The association between sex- and age-dependent vulnerability to neuropsychiatric disorders has been suggested to relate to immaturity at birth in addition to immune activation in the brain, including complex interactions between sex hormones, brain transcriptome, activation of glial cells, and cytokine production (for review see (Ardalan et al., 2019). Thus, our study showing sex differences in expression of genes involved in cognitive processes and inflammatory/redox signalling support the notion that there may be sex-dependent differences in neurocognitive deficits after exposure to environmental toxicants during pregnancy and lactation.

4.5. Neurobehavioral effects in maternally POP-exposed offspring

We have previously reported that moderate X-ray exposure restored learning and memory deficits (as measured in the Barnes maze) in C57BL/6NTac 8-oxoguanine DNA glycosylase 1 (Ogg1)+/− (heterozygote) mice (Hofer et al., 2018), while exposure to algae toxins in adult mice did not affect performances in the Barnes maze or the open field test (Myhre et al., 2018a). Despite clear effects and similarity in gene expression between exposure groups in the present study, this was not manifested in equally clear effects on behaviour outcomes in the Barnes maze. Only two significant learning and memory outcomes (not moving, escape latency) were affected in high exposed offspring subjected to a stressful environment. Interestingly, mediation analysis indicated that changes in gene expression to some extent brought behaviour outcomes back to the normal situation. This may have impaired expression of exposure related behaviour effects in the Barnes maze.

The results on behavioural outcomes are to some extent contradictory to results obtained in human observational studies. Adverse neurodevelopmental effects are reported after exposure to OC compounds (Urabe et al., 1979; Kilburn and Thornton, 1995; Schantz, 1996; Winneke et al., 1998; Ribas-Fito et al., 2003, Ribas-Fito et al., 2007; Eskenazi et al., 2006; Korrick and Sagiv, 2008; Park et al., 2010; Torres-Sanchez et al., 2013), PCBs (Fein et al., 1984; Rogan et al., 1986; Gladen et al., 1988; Tilson et al., 1990; Jacobson and Jacobson, 1996; Seegal, 1996; Longnecker et al., 1997), and BDEs (Roze et al., 2009; Herbstman et al., 2010; Gascon et al., 2011; Cowell et al., 2015). The epidemiological evidence for adverse neurodevelopmental effects of PFASs seems less convincing compared to chlorinated and brominated POPs, and some results are contradictory. For example, PFOA exposure in children were associated with increased reading ability and reduced hyperactivity (Quaak et al., 2016; Zhang et al., 2018), cognitive dysfunction, language processing and social developmental abilities, as well as perturbing the fine and gross motor abilities (Goudarzi et al., 2016). Prenatal exposure to PFNA has been shown to decrease scores in tests related to verbal reasoning (Wang et al., 2015), and disturbances related to impulsivity (Gump et al., 2011). In a recent cohort study, no consistent evidence was found to conclude that prenatal exposure to PFASs (PFHpS, PFOS, PFHxS, PFOA, PFDA, PFUnDA and PFNA) are associated with ADHD symptoms or cognitive dysfunctions in preschool children aged three and a half years (Skogheim et al., 2020). The results showed negative relationships with nonverbal working memory, however, on the other hand positive relationships with verbal working memory. The relationships were weak which suggests no clear association and according to the authors a need for replication (Skogheim et al., 2020).

Animal studies support epidemiological associations in humans reported between pre- and postnatal POP exposure and neurobehavioral effects. Studies of PCB exposure during both gestation and lactation indicate behavioural effects in rodents (for review, see (Eriksson and Fredriksson, 1996b, a; Holene et al., 1998; Berger et al., 2001; Branchi et al., 2005; Mariussen and Fonnum, 2006) and monkeys (Rice, 1999). Mice or rats pre- or postnatally exposed to brominated POPs exhibited learning and memory deficits (Viberg et al., 2003; Eriksson et al., 2006; Koenig et al., 2012; Sun et al., 2017). To our knowledge, developmental neurobehavioral effects are not previously reported for the highly brominated BDE-100 or BDE-207 which are found at high levels in the offspring brain in the present study. Other hippocampus-associated effects have been reported for brominated POPs, like disturbed long-term potentiation (LTP) (Dingemans et al., 2007) and disturbed sensorimotor behaviours in neonates (Miller-Rhodes et al., 2014).

Recent gene-expression studies in rat cerebellar granular neurons using a sub-toxic and marginally toxic concentration of a POP mixture with similar composition as the one used in the present study, revealed differential expression of genes involved in apoptosis, oxidative stress, neurotransmission and cerebellar development, with more genes affected at the marginally toxic concentration (Berntsen et al., 2021). Additionally, we observed increased proliferation and decreased synaptogenesis at human relevant concentrations in human neuronal stem cells using the same POP mixture (Davidsen et al., 2021).

The POP mixture also increased swimming speed of larval zebrafish (Khezri et al., 2017a). This behavioural effect was similar to that observed with perfluorooctanesulfonic acid (PFOS), although the gene expression profile differed between exposures. Some studies report lack of effects on learning and memory after PFOS exposure (Lau et al., 2003), while others reported increased motor activity and reduced habituation in gestationally and lactationally exposed neonatal rats (Butenhoff et al., 2009). Johansson and collaborators exposed mice to single doses of PFOS, PFOA and PFDA, and effects on spontaneous behaviour and habituation were observed with dose-response trends (Johansson et al., 2008). In another study, adult mice exhibited a dose dependent disturbed locomotor activity following a single neonatal exposure to PFHxS (Viberg et al., 2013).

To our knowledge, very few studies have investigated how POP exposure changes behaviour to an acute stressor, however, similarly exposed siblings of the mice in the current study were subjected to a restraint stress test in which male mice from the high exposed group showed an elevated corticosterone response compared to controls (Hudecova et al., 2018). This shows that exposure can elicit adverse reactions to stressors. Other studies have reported effects of combined POP and stress exposure on behavioural outcomes. In these studies, exposure to both PFOS and stress occurred in pregnant mice, thus before behaviour was tested (Fuentes et al., 2006, 2007a, 2007b; Ribes et al., 2010). In detail, Fuentes and colleagues reported that prenatal mortality in mice concurrently exposed to PFOS and restraint was higher than in unrestrained mice exposed to PFOS (Fuentes et al., 2006). The same group further found that mice prenatally exposed to PFOS and restraint exhibited a reduced mobility in the open-field test (Fuentes et al., 2007a). In the Morris water maze test an interaction between sex and restraint was observed with a worse learning rate in female mice born from dams concurrently exposed to PFOS and restraint (Fuentes et al., 2007a). In another study, PFOS and maternal stress interaction did not cause adverse effects on physical maturation or in neuromotor development, however, it caused a reduced distance travelled in the open-field test in adult offspring (mainly observed in females) indicating long lasting functional alterations (Fuentes et al., 2007b). In Ribes et al., mice prenatally exposed to PFOS spent more time in the centre of an open field when compared to the prenatally stressed mice, while no sex differences or interactions between PFOS exposure and restrain were reported (Ribes et al., 2010). The findings reported by (Fuentes et al., 2006, 2007a, 2007b; Ribes et al., 2010) support the present study since the maternally exposed mice exhibited spatial learning and memory deficits in the Barnes maze when subjected to air flow stress from a fan.

4.6. Statistical approaches used to estimate predictors for neurobehavioral outcomes

The present results also illustrate how the variable importance in RF differs from traditional variable selection procedures. When several variables are highly collinear (Fig. 4, Supplementary Fig. S4) but good predictors of the response, as are the expression of hippocampal genes, stepwise and criterion-based variable selection procedures will typically retain one or two of the collinear variables but discard the rest. In contrast, RF "spreads" the variable importance across all explanatory variables. This approach guards against the elimination of variables which are good predictors of the response, and may be biologically important (Cutler et al., 2007).

5. Conclusions

We conclude that, despite species differences, the POP mixture used in the present study is useful for realistic human exposure-scenario studies that aim to increase the understanding of how defined, complex mixtures affect biological functions in females and their developmentally exposed offspring. Human relevant plasma and brain concentrations were obtained in low dose offspring and was associated with changes in hippocampal gene expression relevant to brain function. Behavioural endpoints obtained in the Barnes maze were less affected by POP exposure.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130123.

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