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Peripherally administered persistent organic pollutants distribute to the brain of developing chicken embryo in concentrations relevant for human exposure

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ABSTRACT

Persistent organic pollutants (POPs) can reach the fetal brain and contribute to developmental neurotoxicity. To explore the distribution of POPs to the fetal brain, we exposed chicken embryos to a POP mixture, containing 29 different compounds with concentrations based on blood levels measured in the Scandinavian human population. The mixture was injected into the allantois at embryonic day 13 (E13), aiming at a theoretical concentration of 10 times human blood levels. POPs concentrations in the brain were measured at 0.5, 1, 2, 4, 6, 24, 48, and 72 h after administration. Twenty-seven of the individual compounds were detected during at least one of the time-points analyzed. Generally, the concentrations of most of the measured compounds were within the order of magnitude of those reported in human brain samples. Differences in the speed of distribution to the brain were observed between the per- and polyfluoroalkyl substances (PFASs), which have protein binding potential, and the lipophilic polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and brominated flame retardants (BFRs). Based on pharmacokinetic modeling, PFASs were best described by a one compartment model. PFASs displayed relatively slow elimination *(Kel)* and persisted at high levels in the brain. Lipophilic OCPs and PCBs could be fitted to a 2-compartment model. These showed high levels in the brain relative to the dose administrated as calculated by area under the curve (AUC)/Dose. Altogether, our study showed that chicken is a suitable model to explore the distribution of POPs into the developing brain at concentrations which are relevant for humans.

1. Introduction

Persistent organic pollutants (POPs) are halogenated industrial chemicals highly resistant to environmental degradation. Worldwide concern exists because of their tendency to bioaccumulate in living organisms. Exposure to POPs has been associated with a variety of adverse health effects. These include cancer, allergies and hypersensitivity, reproductive toxicity, alterations of the hormonal system as well as effects on the nervous and immune systems (Secretariat of the Stockholm Convention, 2019b). POPs include chlorinated, brominated,

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and per- and polyfluoroalkyl substances (PFASs) (Secretariat of the Stockholm Convention, 2019a). Chlorinated and brominated POPs are predominantly distributed in lipid rich tissues such as adipose tissue and blood lipids, whereas PFASs are associated with proteins, and are found in the highest concentrations in liver, kidney, and blood (Karrman et al., 2006; Lau, 2015). All three classes of compounds have been detected in the human brain (Dewailly et al., 1999; Maestri et al., 2006; Mitchell et al., 2012; Pérez et al., 2013). The accumulation of POPs in the body starts *in utero*, through maternal exposure to POPs, and later continues via contaminated food, air or water (WHO, 2008). Several industrial compounds including POPs can transfer to the fetus via the placenta and to the infant through breast milk. The blood brain barrier (BBB) protects only partially against the entry of these compounds into the brain (Grandjean and Landrigan, 2014), and POPs are detected in the brain of human fetuses (Mamsen et al., 2019). This results in exposure to these chemicals in early life and potentially adverse effects on brain development, which may lead to neurodevelopmental disorders such as cognitive and neurobehavioral impairment (Grandjean and Landrigan, 2014).

We have previously designed an environmentally relevant mixture of POPs for use in animal and *in vitro* experimental studies, containing 29 different chlorinated, brominated, and PFASs (Berntsen et al., 2017a). The mixture contains POPs at concentrations based on those measured in human blood in Scandinavia, and is aimed to provide a defined and realistic mixture of environmental contaminants for toxicity studies that could reflect the relative levels of POPs to which the general human population are exposed (Berntsen et al., 2017a).

The abundance of potential hazardous chemicals in the brain is of special relevance during development, as they can cause developmental neurotoxicity (DNT) by altering its normal tissue architecture and/or molecular mechanisms. Studies *in vitro* and *in vivo* in animal models have reported neurodevelopmental effects caused by polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) (Fonnum and Mariussen, 2009), or PFASs (Berntsen et al., 2017b, 2018; Berntsen et al., 2020; Yadav et al., 2021b). In our recent study we found transfer of POPs to mice offspring brains after maternal exposure with a mixture of similar composition as the one used in chicken embryos, resulting in gene expression changes in hippocampus related to brain function (Myhre et al., 2021). In addition, the same POP mixture showed adverse effects on neuronal cell function and neurodevelopmental processes vital for normal brain development (Berntsen et al., 2020; Davidsen et al., 2021; Yadav et al., 2021a). Human epidemiological studies have also shown associations between POP exposure and neurodevelopmental effects. For example, in a multi-pollutant analysis of 27 POPs in a Norwegian birth cohort study, early-life exposure to β-hexachlorocyclohexane (β-HCH) and perfluorooctanesulfonic acid (PFOS) was associated with increased risk of attention-deficit hyperactivity disorder (ADHD) (Lenters et al., 2019). Further, Sagiv et al. (2012) reported an association between organochlorines and neuropsychological measures of attention among 8 year old children prenatally exposed to organochlorines. In another study, they found significant associations of prenatal exposure to polybrominated diphenyl ethers (PBDEs) with poorer attention and executive function later in childhood (Sagiv et al., 2015).

Information about which individual POPs reach the developing fetal brain and at what final concentrations, would allow estimation of the final exposure and the associated potential neurotoxic consequences. The chicken egg offers a simple experimental embryo model, without maternal distribution and mother-embryo transfer, that can be useful for gathering such information. In the present study, we injected a defined mixture of POPs (Berntsen et al., 2017a) into chicken eggs and measured their distribution to the developing fetal brain. Several aspects of the stages of neuronal development in chickens are well characterized (Bjornstad et al., 2015). Chicken cerebellar development from embryonic day 12 (E12) - E21 roughly corresponds to the last gestational trimester and first postnatal year in humans (Abrahám et al., 2001; Austdal et al., 2016; Bjornstad et al., 2015; Volpe, 2009). The chicken cerebellum enters a growth spurt around E13 that lasts until around E17 and after E17 cerebellar growth continues decelerating (Austdal et al., 2016). Chicken embryos (from E13-E17) have previously been used as an animal model to study brain development after exposure to POPs (Berntsen et al., 2020; Yadav et al., 2021b), different pharmaceuticals (Aden et al., 2008; Austdal et al., 2016; Fjelldal et al., 2019) and environmental toxicants (Mathisen et al., 2013). The development of the chorioallantoic membrane (CAM) is fully differentiated by E13 (Nowak-Sliwinska et al., 2014), whereas the BBB in chicken matures between E10 and E16 (Stewart and Wiley, 1981). Injection experiments using horseradish peroxidase into the allantoic vein of chick embryos indicated a decreasing permeability of the brain blood vessels from E13 of development onwards (Wakai and Hirokawa, 1978), suggesting maturation of the BBB.

A recent study from our group showed that chicken embryo is a relevant animal model to study drug distribution to the brain at different developmental stages. We found that the drugs examined, two different antiepileptics, rapidly distributed to the brain as early as 5 min after a single injection into the allantois of the egg at E13 and reached the CNS in human-relevant concentrations (Zosen et al., 2021). In the present study we used the same approach and chose to inject eggs at E13 when the BBB and CNS still are not well developed (Bjornstad et al., 2015), roughly corresponding to the third trimester in humans (Haddad-Tóvolli et al., 2017). This exposure site was chosen to allow the compounds to reach the brain through the systemic blood circulation thereby mimicking what happens during early stages of development in humans. We predict that injected pollutants will distribute in the allantoic fluid, be absorbed through CAM, and distribute to the brain via the blood stream, followed by elimination from the brain (Prediction 1).

Development of the BBB and maturation of barrier transporter systems are vital for protection of the fetal brain from exposure to toxic substances, excluding them from the fetal CNS (Goasdoué et al., 2017). At the BBB, there are several members of the ABC (ATP-binding cassette) transporters, e.g., P-gp (permeability glycoprotein) and MRP (multidrug resistance proteins), which control the passage of a wide range of endogenous or xenobiotics substrates (Terasaki and Ohtsuki, 2005). Several of these transporters are expressed in different tissues, including the brain of the chicken (Haritova et al., 2010). To our knowledge, no study has been reported so far on POPs as substrates for BBB transporters. However, it has been shown that that environmental toxicants, like PCBs, dioxins, and BFRs, can compromise BBB function by targeting the activity of the important transporter P-gp (Trexler et al., 2019; Wang et al., 2011a). It is expected that small lipid soluble compounds such as chlorinated and brominated POPs will readily diffuse across the BBB. Other compounds, such as protein binding PFASs would need carrier- or receptor-mediated transport (Goasdoué et al., 2017). In general, PFASs do not readily cross the mature BBB since the levels of perfluorooctanoic acid (PFOA) and PFOS in the cerebral spinal fluid in adult humans were about 1% of those in serum (Harada et al., 2007). However, there may be potential for some PFASs to cross the immature blood-brain barrier (Borg et al., 2010; Chang et al., 2009; Ishida et al., 2017). Based on this we predict that concentrations of the compounds reaching the brain will depend on the chemical property of the individual compounds (Prediction 2).

Furthermore, we assume that although POPs are administered as a single injection of a highly concentrated mixture, their dilution into the egg volume and subsequent distribution will result in concentrations of individual compounds in the brain that are in the human relevant exposure range (Prediction 3).

We also postulate that distribution to the developing chicken fetal brain would follow standard pharmacokinetics (Prediction 4), which could be modelled using pharmacokinetic software initially developed for human application. This software has previously been applied to calculate the different pharmacokinetic parameters determining the levels of drugs in blood and brain in other species e.g., mice (Andersen

et al., 2009; Boix et al., 2013).

2. Material and methods

2.1. POP mixture

The POP mixture was designed and prepared at the Norwegian University of Life Sciences (NMBU), Oslo, Norway (Berntsen et al., 2017a). The mixture contained 29 different compounds (Supplementary Table 1), including six PFASs (perfluorohexanesulfonic acid (PFHxS), PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA)); seven brominated (Br) compounds (PBDE 47, PBDE 99, PBDE 100, PBDE 153, PBDE 154, PBDE 209, and hexabromocyclododecane (HBCD)); and sixteen chlorinated (Cl) compounds (PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180, *p,p'*-dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), α-chlordane, oxychlordane, *trans*-nonachlor, α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH (lindane) and dieldrin). The compounds were selected from their respective compound groups based on prevalence in blood, breastmilk and/or food, and their relative concentrations based on Scandinavian human blood levels. The stocks used in the present study were dissolved in DMSO at a concentration of 10^6 times blood levels and stored in glass vials at −80 °C (Berntsen et al., 2017a).

2.2. Injection of chicken eggs and exposure of developing embryos to POPs

Fertilized chicken (*Gallus gallus*) eggs, weighing 50–55 g, were obtained from Nortura Samvirkekylling (Våler, Norway) and incubated at 37.5 °C in 45% relative humidity in an OvaEasy 380 Advance EXII Incubator (Brinsea,Weston-super-Mare, UK). Sex determination of embryos was not performed. On E13, prior to injection, eggs were weighed and trans-illuminated with a LED lamp (Brinsea) to visualize spontaneous movements confirming living embryos. For administration, the POP mixture stock was diluted 1/100 in saline and with a 29-gauge needle injected through the CAM into the allantois of each egg (1 μL saline solution/gram egg weight). Injection was guided by trans-illumination with the LED lamp to avoid injecting into blood vessels. The administration resulted in a final exposure concentration of 10x (times) human blood levels, assuming uniform distribution of the compounds throughout all compartments of the egg. Each egg received only a single administration of the POP mixture. At 0.5, 1, 2, 4, 6, 24, 48, or 72 h after administration, the embryos were anesthetized by hypothermia by submerging the eggs in crushed ice for 7 min, hatched, and immediately decapitated. The skull was opened along the cranial sutures and the cranium was removed to expose the brain. The whole brain was isolated with a spatula and the meninges were removed with forceps. The brains were snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Two or three eggs were injected per sampling time-point. Animals were handled in accordance with the Norwegian Animal Welfare Act and the EU directive 2010/63/EU, and the study was approved by the Norwegian Food Safety Authority (application ID: FOTS 13896). The exposure scenario is shown in Fig. 1.

2.3. Measurement of POPs concentration in chicken embryo brains

PFASs were analyzed in each individual embryo brain, ensuing in three analysis results (n = 3) for each time point (Table 1A). OCPs, PCBs and BFRs were analyzed in brains from a different set of exposures and required two brains pooled for a single chemical quantification, resulting in only one analysis result (n = 1) per time point (Table 1B).

Analysis of brain samples for PFASs was performed at the Department of Environmental Health at the Norwegian Institute of Public Health (Oslo, Norway) as described previously (Haug et al., 2009). In short, PFASs were extracted with methanol. Concentrations of PFASs were determined using column-switching liquid chromatography (LC) coupled to a triple-quadrupole mass spectrometer (MS).

The rest of the compounds (PCBs, PBDEs and OCPs) were analyzed at the Norwegian University of Life Sciences (NMBU), Department of Food Safety and Infection Biology, Laboratory of Environmental Toxicology. Extractions were performed with cyclohexane/acetone and water. It was followed by gel permeation column or sulphuric acid for 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 PBDEs and HBCD was performed on a HRGC–LRMS (Polder et al., 2014). Details from the extraction, clean-up and instrument run for the samples and quality control parameters can be found in Supplementary material.

2.4. Pharmacokinetics modelling

We applied the pharmacokinetic software package Kinetica 5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) to model the distribution to the embryonic chicken brain considered as an extravascular compartment. A one-compartment or two-compartment extravascular pharmacokinetic model with or without lag was selected based on the lowest Akaike's Information Criteria and subsequent visual inspection of the fitted curves. This allowed us to calculate the pharmacokinetic distribution parameters (Table 2) based on the concentrations curves and the absolute dose injected. The ratio between the area under the curve (AUC) and dose (*AUC/Dose*) was also calculated. The log of the n-octanol/water partition coefficient (*logKow*), defined as the ratio of the concentration of a chemical in n-octanol and water at equilibrium at a specified temperature, was obtained from available public sources (referenced in Table 2).

3. Results

3.1. Concentrations of POP mixture compounds in chicken embryo brain

First, PFASs were quantified for each time-point. PFOS reached maximum concentrations at 6 h after injection, whereas the other PFASs reached maximum concentration at 24 and 48 h after injection. The

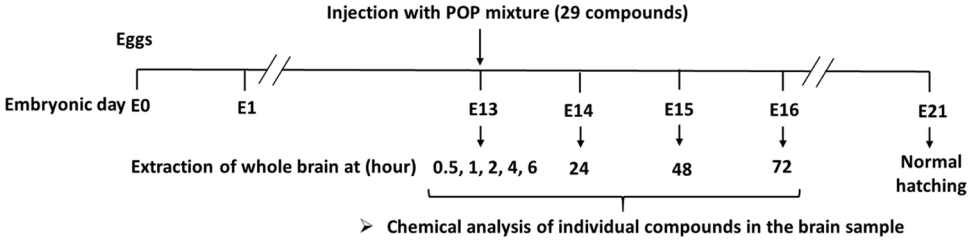

Fig. 1. Illustration of the experimental timeline. The POP mixture (29 compounds) was administrated by injection through CAM into the allantois at embryonic day (E) 13. At 0.5, 1, 2, 4, 6, 24, 48 and 72 h after administration, the whole brain was isolated and chemical analysis of individual compounds in the brain was performed.

Table 1
Chemical concentrations of POP mixture in chicken embryo brain. Chicken embryos on E13 were exposed with a POP mixture containing 29 compounds resulting in a final concentration equivalent to 10x human blood levels in the egg. After 0.5, 1, 2, 4, 6, 24, 28 and 72 h exposure individual compounds were analyzed in embryonic brains. A) Measured concentrations represent the mean value ± SEM of PFASs of three brains at each time point (n = 3); B) Measured concentrations represent single measurements from two pooled brains of OCPs, PCBs and BFRs at each time-point.

Individual Compounds in POP mixture	Detection limit	Time h							
A) ng/g wet weight		0.5	1	2	4	6	24	48	72
PFASs*									
PFHxS	0.50	nd	nd	nd	3.52 ± 0.26	8.60 ± 1.90	21.19 ± 3.05	20.64 ± 1.67	14.74 ± 0.83
PFOS	0.50	5.10 ± 0.29	1.54 ± 0.36	25.74 ± 3.51	77.57 ± 10.55	201.81 ± 25.36	190.89 ± 28.40	132.45 ± 8.04	83.18 ± 6.29
PFOA	0.50	#0.15 ± 0.00	nd	nd	0.07 ± 0.02	0.72 ± 0.25	3.70 ± 1.09	5.79 ± 0.87	5.23 ± 0.51
PFNA	0.50	nd	nd	nd	0.93 ± 0.14	1.54 ± 0.21	2.70 ± 0.39	3.00 ± 0.48	1.66 ± 0.24
PFDA	0.50	nd	nd	nd	0.96 ± 0.18	2.02 ± 0.37	2.20 ± 0.22	2.62 ± 0.07	1.53 ± 0.10
PFUnDA	0.50	0.10 ± 0.02	0.05 ± 0.00	0.67 ± 0.09	1.58 ± 0.13	3.48 ± 0.46	3.60 ± 0.41	4.26 ± 0.32	3.26 ± 0.31
B) ng/g of lipid									
OCPs									
HCB	0.01	50.00	26.84	6.92	19.63	11.07	2.92	2.57	1.67
α-HCH	0.03	4.21	nd	nd	nd	nd	nd	nd	nd
β-HCH	0.05	38.42	12.11	5.39	5.56	3.93	nd	nd	nd
γ-HCH	0.04	10.53	nd	nd	2.22	nd	nd	nd	nd
Oxychlordane	0.02	14.21	7.90	nd	5.56	2.50	nd	nd	nd
alpha-chlordane	0.02	7.90	4.74	3.46	4.82	2.50	nd	nd	nd
trans-Nonachlor	0.01	18.95	13.16	15.39	22.96	13.93	1.83	nd	nd
p,p'-DDE	0.11	154.21	139.47	164.23	354.07	205.00	26.27	18.29	11.67
Dieldrin	0.40	40.00	nd	nd	22.22	19.29	nd	nd	nd
PCBs									
PCB-101	0.04	nd	5.26	5.39	10.37	5.36	nd	nd	nd
PCB-118	0.01	12.28	10.70	18.59	46.79	28.33	3.41	2.10	1.94
PCB-138	0.01	17.90	17.37	59.23	223.33	170.71	20.07	10.00	7.71
PCB-153	0.01	19.83	20.35	82.95	364.32	297.38	36.73	19.91	15.76
PCB-180	0.01	1.75	1.75	15.13	129.01	149.05	50.48	19.52	11.32
BFRs									
PBDE-47	0.01	2.11	1.58	1.54	5.56	3.21	nd	nd	nd
PBDE-99	0.02	nd	2.11	0.77	2.96	1.79	nd	nd	nd
PBDE-100	0.01	1.05	1.58	0.77	2.22	1.79	3.70	0.86	nd
PBDE-153	0.02	1.05	na	1.15	1.11	nd	nd	nd	nd
PBDE-154	0.02	1.58	1.05	nd	0.74	1.43	nd	nd	nd
PBDE-209	0.11	nd	nd	nd	nd	nd	nd	nd	4.79
HBCD	0.11	nd	6.33	nd	10.14	nd	nd	nd	nd

Abbreviations: nd (not detected); PCB (polychlorinated biphenyls); OCP (organochlorine pesticides); BFR (brominated flame retardants); PFASs (per- and poly-fluoroalkyl substances). *Limit of quantitation (LOQ): about 0.5 pg/mg, results were indicated below the LOQ when the chromatogram showed a distinct peak. #0.15 ± 0.00 detected in only one sample.

Table 2
Dose administered, pharmacokinetics parameters in the brain of chicken embryos, and logKow of selected POPs injected into the allantois of the chicken egg.

Compound	Model	Dose	Ka	lag	Kel	AUC	MRT	Cmax Calc	Tmax calc	AUC Dose	logKow
		ng	h-1	h	h-1	(h)*(ng/μL)	h	ng/g	h		
PFASs											
PFOS	1 C - lag	12291.40	0.60	3.41	0.017	15750.70	56.33	237.79	9.58	1.28	6.43 (Wang et al., 2011b)
PFHxS	1 C - lag	1886.48	0.06	2.00	0.016	2234.02	61.47	22.30	30.98	1.18	5.17 (Wang et al., 2011b)
PFOA	1 C - lag	958.62	0.02	3.69	0.017	810.21	54.75	5.56	57.04	0.84	5.30 (Wang et al., 2011b)
PFUnDA	1 C - lag	104.49	0.29	1.54	0.003	1585.80	377.61	4.00	18.01	15.18	7.15 (Wang et al., 2011b)
OCPs											
p,p'-DDE	2 C - lag	186.44	0.63	1.68	0.266	3786.76	35.29	377.45	3.33	20.31	6.51 (Desban et al., 1989)
HCB	2 C - no lag	35.74	3.74		0.404	349.35	26.20	57.96	0.30	9.77	5.47 (Tolls et al., 2003)
PCBs											
PCB153	2 C - lag	138.60	1.12	1.84	0.085	5969.54	58.61	364.93	3.86	43.07	6.87 (Li et al., 2003)
PCB138	2 C - lag	85,25	1.58	1.86	0.093	3189.71	47.67	229.14	3.47	37.42	7.22 (Li et al., 2003)
PCB180	2 C - lag	73.71	0.54	1.86	0.054	3847.56	32.62	149.05	6.00	52.20	7.16 (Li et al., 2003)
PCB118	2 C - lag	24.76	0.60	1.72	0.244	535.14	45.31	48.89	3.43	21.61	6.69 (Li et al., 2003)

Model: Pharmacokinetic model best fitting concentrations of POP in the chicken brain. *Dose*: Absolute dose injected in allantoic fluid. *Ka*: Absorption rate constant from injection site. *Lag*: Time taken to appear in the brain following administration. *Kel*: Elimination rate constant from brain. *AUC*: Area under the curve. *MRT*: Mean residence time (Time spend by molecules in the brain). *Cmax calc*: Theoretical calculated maximal concentration. *Tmax calc*: Theoretical calculated time at which Cmax is achieved. *AUC/Dose*: Ratio between AUC and dose administered. *logKow*: Logarithm of the n-octanol/water partition coefficient (Kow) as obtained from the literature (references in brackets).

highest concentration was for PFOS (201.81 ± 25.36 ng/g) at 6 h, followed by PFHxS (21.19 ± 3.05 ng/g) at 24 h post injection (Table 1A).

These observations, based on 3 biological replicates and summarized in Supplementary Table 3, also indicate consistency and reproducibility of the injections. For example, at 6 h the SEM is 12.57% of the mean for PFOS, and 13.21% for PFUnDA. Data for the remaining compounds were therefore based on a single measurement in two pooled brains at each time-point (Table 1B).

Of the 29 compounds, 27 were found in the brain samples, and only PCB 28 and PCB 52 could not be detected. Most compounds were already detectable in the brain 0.5 h after injection into the allantois (Table 1B). Variability in the time reaching the maximum brain concentrations between compounds was observed. The OCPs were at their maximum 0.5-1 h after exposure, except *trans*-nonachlor and *p,p′*-DDE which, like the PCBs, peaked at 4–6 h. Other lipophilic compounds (BFRs) peaked between 0.5 and 6 h. Interestingly, the peak concentrations were lower than what would have been expected with a uniform distribution throughout the egg, consistent with barriers preventing free diffusion and emphasizing the importance of such analyses. After reaching the maximum peak, the brain concentrations of OCPs declined rapidly and only DDE was above the detection limit at 72 h post exposure. BFRs had intermediate elimination, whereas PFASs persisted at relative high levels in the brain for relative long times. The levels of PCBs were still detectable but relatively low whereas the levels of PFASs were all relatively high at the end of the study (72 h) compared to the maximum peak. The levels of PBDEs were under the detection limit at 72 h post exposure.

3.2. Pharmacokinetic (PK) modelling

The experimental data in Table 1 seemed to follow different distribution patterns depending on the chemical class of compound. We used an available pharmacokinetic package to derive pharmacokinetic parameters based on the concentrations measured in the brain. Based on these, PFASs showed a good fit to a one-compartment model with lag, OCPs fitted predominantly to a 2 compartments model, and PCBs to a two-compartments model with lag (Table 2 and Figs. 2–4).

PFASs showed lower rates of transfer (*Ka*) from the injection site to the brain compared to the PCBs. The two OCPs, DDE and HCB, modelled showed different transfer rates to the brain. On the other hand, a relationship between the elimination rate constant (*Kel)* and the different classes of POPs was revealed. Thus, whereas the OCPs showed the highest elimination rates, being therefore the compounds disappearing fastest from the embryo brain, PFASs were the ones with the lowest *Kel*, showing relatively high concentrations still 72 h after administration, with data for PCBs in-between those two. The ratios of AUC/Dose were also closely related to compound class, with PCBs showing much higher AUC/Dose ratios than PFASs, while data for OCPs were in between. Reported logKow values (Table 2) are the highest for PCBs whereas OCPs and PFASs have lower values.

Despite the relations between type of compounds and pharmacokinetic parameters, there were also obvious differences within compound classes. PFUnDA had much lower *Kel* than the other PFASs, which also reflected in its residence time and its AUC/Dose, whereas the two OCPs analyzed showed a very high discrepancy in their *Ka*.

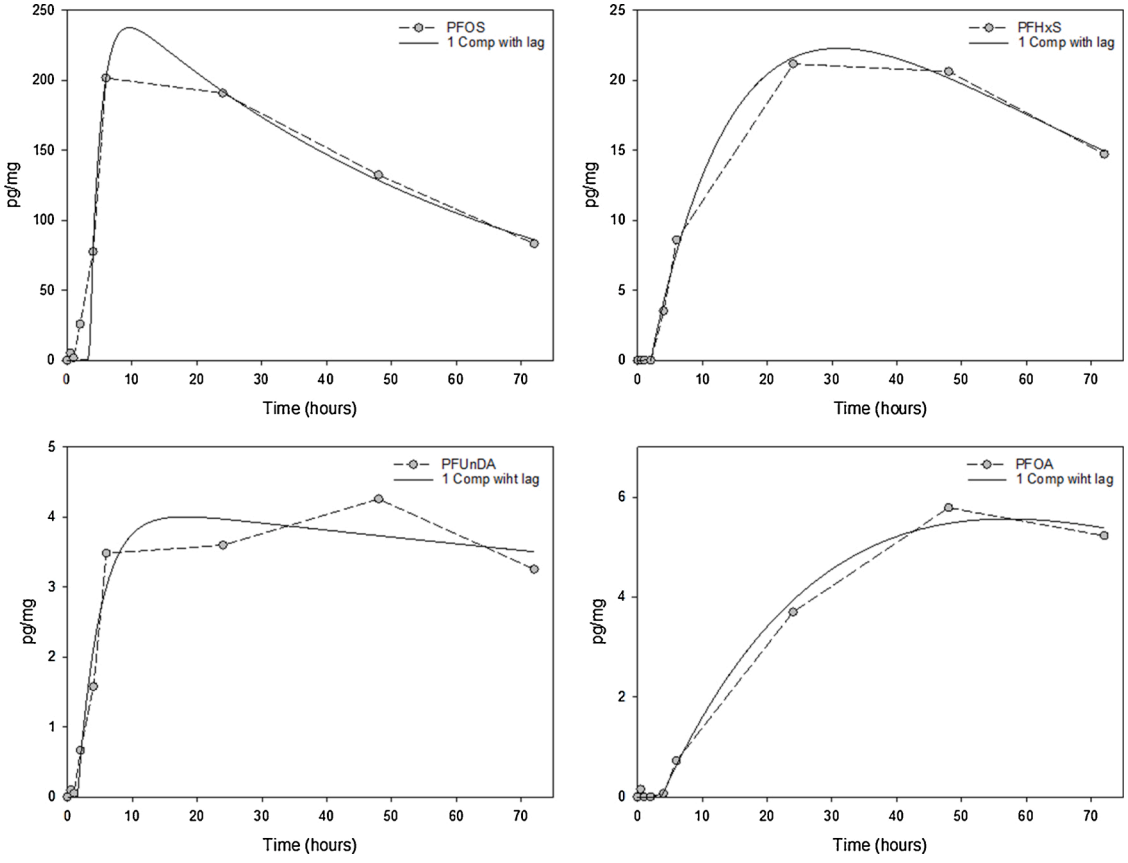

Fig. 2. Mean concentrations (grey circles, dashed line) of PFASs (PFOS, PFHxS, PFUnDA, PFOA,) in the brain of chicken embryos taken at different time-points after their injection in the allantoic fluid of chicken eggs and theoretical values (continuous black spline), calculated in half hour intervals, from the pharmacokinetic model fitted (specified in the legends of the graphs).

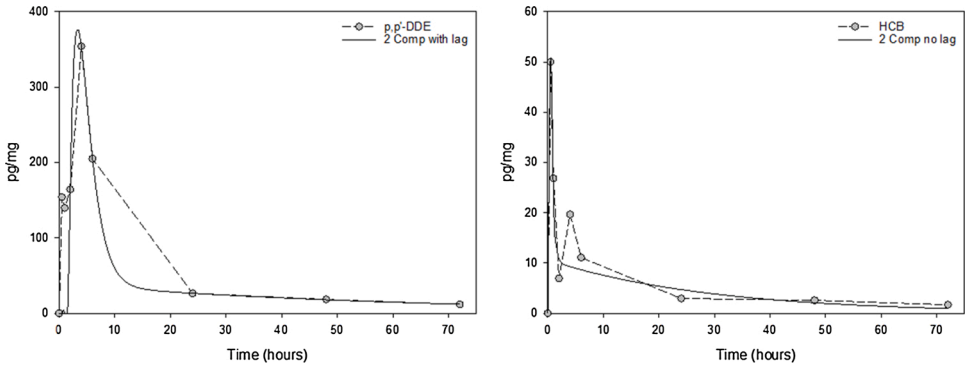

Fig. 3. Concentrations (grey circles, dashed line) of OCPs (*p,p'*-DDE, HCB) in the brain of chicken embryos taken at different time-points after their injection in the allantoic fluid of chicken eggs and theoretical values (continuous black spline), calculated in half hour intervals, from the pharmacokinetic model fitted (specified in the legends of the graphs).

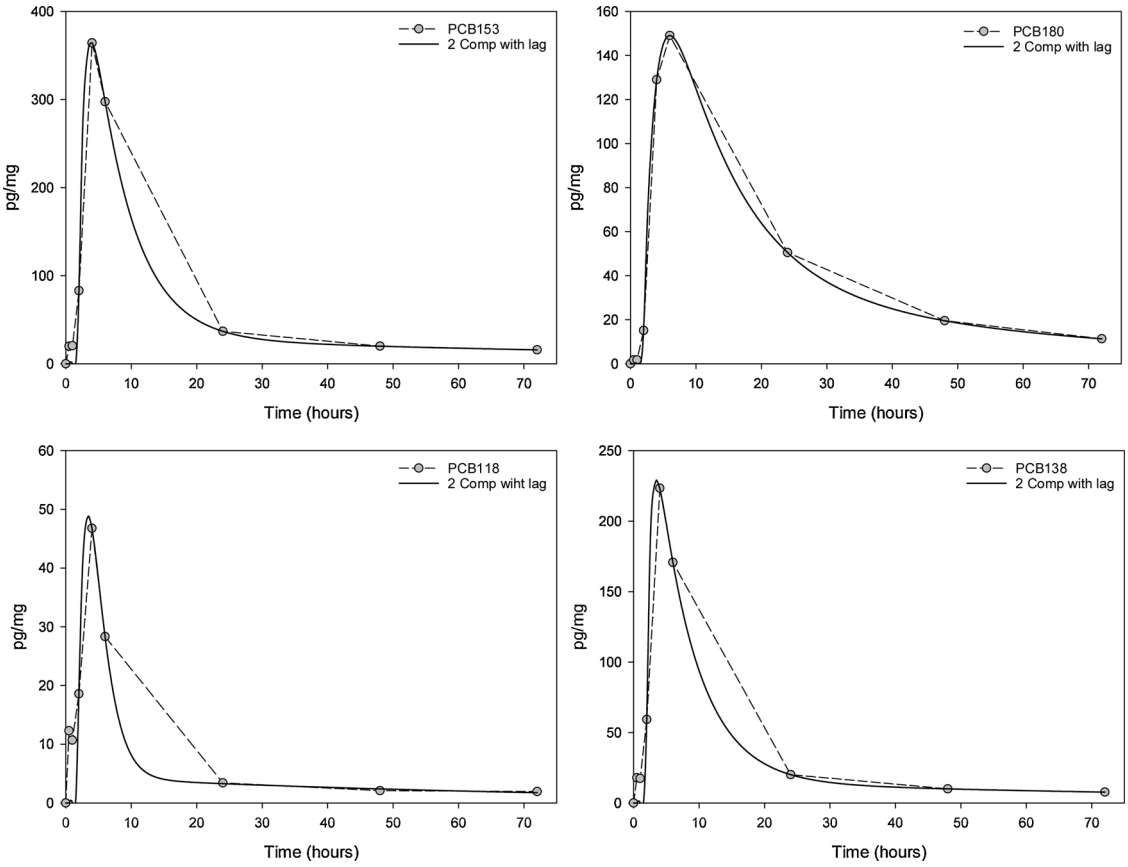

Fig. 4. Concentrations (grey circles, dashed line) of PCBs (PCB 153, PCB 180, PCB 118, PCB 138) in the brain of chicken embryos taken at different time-points after their injection in the allantoic fluid of chicken eggs and theoretical values (continuous black spline), calculated in half hour intervals, from the pharmacokinetic model fitted (specified in the legends of the graphs).

4. Discussion

POPs and different drugs/chemicals have shown to cause neurodevelopmental effects in the chicken embryo model (Austdal et al., 2016; Berntsen et al., 2020; Mathisen et al., 2013; Yadav et al., 2021b). In this study we exposed chicken embryos at E13 with a POP mixture containing 29 compounds resulting in a final concentration equivalent to 10x human blood levels in the egg by injection into the allantois. After administration, 27 of 29 of the individual compounds were detected in the developing brain during at least one of the time-points analyzed. Concentration reached a peak value, followed by a decline over time. This confirms our prediction 1, that the compounds in our mixture reach the embryonic brain and are subject to elimination. The injected compounds showed the regular pharmacokinetic phases: absorption (passage from the administration site to the brain), followed by elimination from the brain. Since the compounds reached the brain few minutes after injection into the allantois, absorption is most likely through the systemic blood stream. Elimination may have many different mechanisms, such as redistribution through the BBB or other barriers of the brain, or metabolism inside the brain. These phases were also observed for drugs using the same route of administration (Zosen et al., 2021).

We observed differences in the speed of distribution to the brain, with the protein binding compounds (PFASs) being slower than the lipophilic (PCBs, OCPs, BFRs), confirming our prediction 2 that the

compounds reaching the brain will depend on the chemical property of the individual compounds.

The concentrations of most of the measured compounds in chicken embryo brains were within the same order of magnitude as those reported in human brain samples (Supplementary Table 2), as postulated in prediction 3. However, the levels of PFOS in the chicken brain were relatively high compared to levels measured in human brain samples. As exposure to POPs is individualistic in nature, certain groups of people may have higher body burden of certain POPs. A Chinese study reported levels of PFOS up to 118000 ng/mL and PFOA up to 32000 ng/mL in serum from occupationally exposed workers (Fu et al., 2016). Although there are few studies reporting levels of PFASs in the human brain, according to Maestri et al. (2006) the concentrations in the brain of PFOS and PFOA can be expected to be the 25% and 17% of their concentrations in serum, respectively. Thus, this would correspond to brain concentrations up to 29500 ng/mL for PFOS and 5440 ng/mL for PFOA in Fu et al. (2016).

The POPs studied can be differentiated based on which pharmacokinetic model fits them best. Thus, whereas pharmacokinetic modelling for PFASs was best fitted by a standard one compartment extravascular model, OCPs (HCB, DDE) and PCBs could be best fitted by a standard two compartmental extravascular model. In pharmacokinetics, multi-compartmental models are used to mathematically describe non-linear kinetics of a drug during the elimination phase, which can be detected using a semilogarithmic plot. Accordingly, the fitting of a one or two compartments model merely expresses if the pharmacokinetics is linear (one compartment) or not (two or more compartments). These compartments have conventionally been associated to anatomical locations in the organism where the drug distributes. However, non-linearity can be due to other physiological processes, for example the kinetics of the metabolic enzymatic reactions (Macheras and Iliadis, 2016). Thus, the compartmental modelling distinguishing PFASs from OCPs and PCBs would imply that probably different physiological mechanisms or routes are responsible for the distribution of these compounds to the brain. Which process, or processes, determines the non-linearity in the distribution of these compounds to the chicken embryo brain demands a more advanced knowledge of this experimental model. OCPs are characterized by fitting to a model without lag, indicating a faster absorption. Indeed, they presented larger absorption rates (Ka). Considering the elimination constant (Kel), OCPs showed a more rapid accumulation followed by a faster elimination from the brain than PCBs compared to the PFASs, which still persisted at relatively high levels at the end of the study (3-day post injection). PFUnDA had the lowest Kel, indicating a mean residence time of more than 300 h. Thus, PFUnDA would stay at relatively significant levels in the brain for at least until hatching. PFUnDA is one of the most abundant PFASs in the brains of polar bears from Greenland (Eggers Pedersen et al., 2015). Also, in brain samples from harbour seals and red-throated divers the longer chained PFASs 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). In addition, we have previously examined the toxicity of each individual PFASs present in the POP mixture in cultured rat cerebellar granule neurons (Berntsen et al., 2017b). We observed that toxicity of PFASs increased with increasing carbon chain length, with PFUnDA being the most potent inducer of cytotoxicity. The longer residence time of this compound and its possible higher neurotoxicity could potentially make this a compound of high concern with respect to developmental neurotoxicity. This concern is strengthened by the fact that production and use of PFUnDA in a multitude of consumer products, is not regulated by any national or international legislation.

The calculated AUC/Dose, giving valuable information about total exposure in the brain in relation to the dose administered, is lowest for PFASs and increases with HCB, DDE and PCBs. This implies that, for the same dose administered, the brain exposure would be highest for PCBs, followed by DDE, HCB and PFASs, except for PFUnDA. This information can be especially useful when considering the potential impact of levels found in humans in the development of the embryonic brain.

The differences in pharmacokinetics between the compounds studied can be related to their lipophilicity. One common measure of lipophilicity is the n-Octanol/Water Partition Coefficient (Kow), which is the ratio of the concentration of a chemical in n-octanol and water at equilibrium. Kow is normally expressed as a logarithm (logKow). LogKow is generally directly related to solubility in fat (as measured in n-octanol in this case), is proportional to the molecular weight of a substance, and can predict the distribution of pollutants in tissues (Hellou et al., 2002). Despite overlaps, the different POPs groups are also clustered by their logKow. On the other hand, a negative, correlation (Pearson's r=-0.5594) with the lag, accompanied by positive correlations with the mean residential time MRT and the AUC/Dose (Pearson's $r = 0.3115$ and $r = 0.7518$ respectively), were observed. This would indicate that the most lipophilic substances are able to get faster into and remain longer in the brain, possibly due to the relative high content of fatty compounds in this organ. For example, highly chlorinated, lipophilic PCBs have among the longest elimination half-lives known in humans, with bioaccumulative and toxic properties (Hofer et al., 2021). However, logKow might not be a good predictor for PFASs as we have to consider their values as uncertain since they are both hydrophobic and oleophobic. Therefore logKow cannot easily be experimentally determined and can only be estimated from their structure (Liu et al., 2019). Together, this indicates that a pharmacokinetic model developed for humans can be applied to the observed concentrations in the chicken brain and can be related to their chemical and physical properties, in line with prediction 4.

The understanding of POPs pharmacokinetics is important for human hazard assessments, since PCB and PBDE concentrations in human brain tissues (Dewailly et al., 1999) have been associated with neurological disorders (Corrigan et al., 1996, 1998; Hatcher-Martin et al., 2012; Mitchell et al., 2012). Such levels were approximately in the same concentration range in maternally exposed mice offspring (exhibiting disturbed hippocampal gene expression) in our recent experiment (Myhre et al., 2021) and in the present study.

In previous studies, different exposure scenarios have been published, e.g., chicken embryos were exposed with POPs during the complete gestation by injection at the early stage *in ovo* or through maternal diets (Death et al., 2021; Wilson et al., 2021; Briels et al., 2018; O'Brien et al., 2009). However, these studies did not focus on the brain as target organ for developmental neurotoxicity. Although humans can be exposed throughout the whole gestation, we have chosen a bolus injection in a time window relevant for the third trimester in human, focussing on distribution to the brain. Our data showed that environmental toxicants injected into the allantois of the egg rapidly distributed to the chicken embryo brain and, at the doses applied, reached the CNS at concentrations which are relevant for humans. Thus, we conclude that this model is suitable for further mechanistic or neurotoxicological studies related to the third trimester. In avian models, as in all other non-mammalian animal models, there are challenges and features which cannot be mimicked correctly when translating the results to a similar setting in mammals. For example, maternal metabolism, placental barrier, and an excretion from eggs are absent in the chicken embryo model, which may cause a prolonged effect of a single exposure (Bjornstad et al., 2015). This last, however, could be an advantage for using the chicken embryo model by reducing the need for multiple injections.

The possibility of interactions among the POPs in the distribution to the brain can be a limitation of the present study, since the experimental design does not allow such assessments. Our aim was to explore the distribution of POPs into the developing brain and establish human relevant concentrations. In general, when chemicals co-occur, they may act additively instead of displaying interactions, currently believed to be the most common scenario, especially at low concentrations (Kortenkamp et al., 2009; Martin et al., 2020). Although less commonly observed, they may also display interactive synergistic (more than additive) or antagonistic (less than additive) effects. In a recent study from

our group, we examined the effects of the same total POP mixture as well as Cl, Br, and PFAAs sub-groups alone, including their combinations, in cultures of rat cerebellar granule neurons (CGNs). We observed that the PFAAs sub-mixture in combination with the Br and/or Cl sub-mixtures exerted a stronger toxic effect than the PFAAs sub-mixture alone, indicating a degree of additivity between the sub-classes (Berntsen et al., 2020). In human neuronal stem cell cultures, additive responses between the different sub-mixtures were generally observed on different developmental neurotoxicity endpoints, although a potentiated effect, in particular by the combination of PFAAs + Br and Br + Cl sub-mixtures, were observed for the end-points synaptogenesis and neurite outgrowth (Davidsen et al., 2021). The same sub-mixtures showed approximately the same tendency for interactions after activation of respiratory burst in human leucocytes *in vitro* (Berntsen et al., 2021). Further studies with the sub-mixtures of the different classes, combinations thereof, and single compounds would be needed to explore the underlying mechanisms of such interactions. In addition, the unknown possible effects of metabolites warrant further studies as well.

5. Conclusion

The results show that individual compounds from a human relevant mixture of POPs injected into the allantois of chicken eggs distribute to the brain of developing embryos, for the most part in concentrations within the same order of magnitude as reported in human brain samples. Differences in the distribution to the brain were observed between the lipophilic PCBs and OCPs compared to the PFASs with protein binding potential. Thus, PCBs showed high exposure in the brain in relation to the dose administered, probably causing a higher CNS toxicity than estimated only from their dose and, likely, blood concentrations alone. This could be related to the physicochemical properties of different classes of compound in the mixture, which should be further investigated. We suggest that POPs reach the chicken embryo brain through the systemic blood circulation thereby mimicking what happens during early stages of development in humans. The present study validates the chicken egg as a valuable animal model to explore the distribution and exposure of POPs in the fetal brain, and its relation to their neurotoxic effects during development, at human relevant concentrations.

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 material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2021.10.013.

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