Influence of prenatal exposure to environmental pollutants on human cord blood levels of glutamate

Aina Palou-Serra^{a,b}, Mario Murcia^{b,c}, Maria-Jose Lopez-Espinosa^{b,c}, Joan O. Grimalt^d,
Eduard Rodríguez-Farré^{a,b}, Ferran Ballester^{b,c,e}, Cristina Suñol^{a,b,*}

^aInstitut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas (IIBB-CSIC); Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); Barcelona, Spain

^bCIBER Epidemiología y Salud Pública (CIBERESP), Spain

^cCenter for Public Health Research (CSISP- FISABIO), Valencia, Spain

^dInstitute of Environmental Assessment and Water Research (IDÆA-CSIC), Barcelona, Spain

^eUniversity of Valencia, Valencia, Spain

*Corresponding Author: Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, IIBB-CSIC-IDIBAPS, Rosselló 161, E-08036 Barcelona, Spain, e-mail: csenqi@iibb.csic.es

e-mail addresses: aina.palou.serra@gmail.com (A. Palou-Serra), murcia_mar@gva.es (M. Murcia), lopez_josesp@gva.es (M.J. Lopez-Espinosa), joan.grimalt@idaea.csic.es (J.O. Grimalt), erfnmc@iibb.csic.es (E. Rodríguez-Farré), ballester_fer@gva.es (F. Ballester), csenqi@iibb.csic.es (C. Suñol)

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ABSTRACT

Some chemicals released into the environment, including mercury and some organochlorine compounds (OCs), are suspected to have a key role on subclinical brain dysfunction in childhood. Alteration of the glutamatergic system may be one mechanistic pathway. We aimed to determine whether mercury and seven OCs, including PCBs 138, 153, and 180, DDT and DDE, hexachlorobenzene (HCB), and beta-hexachlorocyclohexane (β -HCH) influence the cord levels of two excitatory amino acids, glutamate and aspartate. Secondly, we evaluated if this association was mediated by glutamate uptake measured in human placental membranes. The study sample included 40 newborns from a Spanish cohort selected according to cord mercury levels. We determined the content of both amino acids in cord blood samples by means of HPLC and assessed their associations with the contaminants using linear regression analyses, and the effect of the contaminants on glutamate uptake by means of [3 H]-aspartate binding in human placenta samples. PCB138, β -HCH, and the sum of the 3 PCBs and seven OCs showed a significant negative association with glutamate levels (decrease of 51, 24, 56 and 54 %, respectively, in glutamate levels for each 10-fold increase in the contaminant concentration). Mercury did not show a significant correlation neither with glutamate nor aspartate levels in cord blood, however a compensatory effect between T-Hg and both PCB138, and 4,4'-DDE was observed. The organo-metallic derivative methylmercury completely inhibited glutamate uptake in placenta while PCB138 and β -HCH partially inhibited it (IC50 values: $4.9 \pm 0.8 \mu\text{M}$, $14.2 \pm 1.2 \text{nM}$ and $6.9 \pm 2.9 \text{nM}$, respectively). We conclude that some environmental toxicants may alter the glutamate content in the umbilical cord blood, which might underlie alterations in human development.

Key words: methylmercury, polychlorobiphenyl (PCB), organochlorine pesticides, placenta, umbilical cord blood, glutamate, transport, pollutants.

1. Introduction

Social and economic development have been mediated by strong industrial growth involving the synthesis, use and release of large numbers of chemicals many of which are potentially harmful to human health and the environment (Li and Macdonald, 2005; Porta et al., 2012). Several of these chemicals, e.g. lead, mercury, polychlorinated biphenyls (PCBs), arsenic and toluene, have been associated to subclinical brain dysfunction and neurodevelopmental disorders such as poorer cognitive and motor development, attention deficit, hyperactivity, and sensory deficits but the underlying processes leading to these effects are still unknown (Grandjean and Landrigan, 2006). Pollutants come from several different sources, move on the environment and eventually accumulate in the food chain. Daily exposure to environmental persistent pollutants results in detectable levels of metals, PCBs and organochlorine pesticides in woman bodies (Woodruff et al., 2011). During pregnancy, the pollutants accumulated by the women can reach the fetus crossing the placenta. Although the placenta regulates the exchange of nutrients and oxygen between mother and fetus and acts as a barrier of many toxicants, it does not provide protection against certain environmental contaminants (Prouillac and Lecoœur, 2010).

The glutamatergic system is one of the targets of several pollutants in the nervous system (Allen et al., 2001; Briz et al., 2010; Fonfría et al., 2005; Fonnum and Lock, 2004; Hogberg et al., 2010; Piedrafita et al., 2008). Glutamate is a non-essential amino acid that has a key role in metabolic pathways, being important for the normal growth and development of the fetus during pregnancy. Glutamate is produced by the fetal liver using maternal glutamine, and the excess of glutamate from the fetal circuit is removed by glutamate transporters (namely excitatory amino acid transporters: EAAT1, EAAT2 and EAAT3) in placenta (Battaglia, 2000; Noorlander et al., 2004). Glutamate is also the major excitatory neurotransmitter in the central nervous system of mammals and it is present in human fetal

brains at concentrations that did not vary during the third trimester of pregnancy (Girard et al., 2006). It mediates key aspects of normal brain function such as sensory information, motor coordination, emotions and cognition. However, it has neurotoxic effects when it is present in excess (Danbolt, 2001). On the other hand, the amino acid aspartate shares the same uptake transport than glutamate. Although its role as a neurotransmitter has not yet been established, recent studies suggest that it may play neuromodulatory functions at developmental stages by acting on glutamate receptors (Errico et al., 2012; Ota et al., 2012).

Mercury has been reported to inhibit glutamate transport in neural cells and to increase glutamate release into the extracellular space of neural cells and platelets (Allen et al., 2001; Borges et al., 2007; Fonfría et al., 2005). Specific attention should be paid to its organometallic compound, methylmercury, because: i) it has recognized properties as a human neurodevelopmental toxicant (Grandjean and Landrigan, 2006); ii) poisoning incidents with methylmercury have demonstrated the potential of this pollutant as neurotoxicant and its serious health consequences (Castoldi et al., 2008; Ekino et al., 2007; Grandjean et al., 2010; Nakagawa et al., 2002); iii) millions of people are nowadays chronically exposed to this contaminant and there is epidemiological (Marsh et al., 1987; Pinheiro et al., 2007; Yokoo et al., 2003) and experimental (Castoldi et al., 2008; Farina et al., 2009; Vendrell et al., 2007, 2010) evidence of the toxic effects of chronic exposure to this metal especially for the nervous system; and iv) previous results from the INMA birth cohort of Valencia (Spain) showed elevated levels of this pollutant in umbilical cord blood, where 70% of newborns had mercury levels above US EPA recommended level, i.e. 5.8 µg/L of MeHg (Ramon et al., 2008).

Moreover, organochlorine compounds (OCs) such as PCBs and dieldrin have also been described to alter glutamate neurotransmission (Briz et al., 2010; Llansola et al., 2010;

Mariussen and Fonnum, 2001; Piedrafita et al., 2008; Stavenes Andersen et al., 2009), which may lead to neurotoxicity. Prenatal exposure to some of them are of high concern since levels above the limit of quantification have been found in a significant percentage of samples of several mother-child cohorts including the INMA cohort of Valencia (Vizcaino et al., 2010, 2011). Furthermore, neuropsychological studies on children reveal an association between PCB prenatal exposure and impaired neuropsychological and psychomotor development (Cheslack-Postava et al., 2013; Fernández et al., 2012; Forns et al., 2012 a,b; Sagiv et al., 2012, Gascon et al., 2013). It is also important to consider that people are simultaneously exposed to several pollutants through the diet (Arrebola et al. 2011, Llop et al. 2010, Ramon et al. 2008) so the effects induced by the pollutants mixture may be different than the ones observed in experimental works with isolated pollutants.

The aim of the present study is to address whether mercury and some OCs alter glutamate uptake in human placental membranes and, thus, influence the levels of excitatory amino acids in cord blood. For this purpose, in the frame of a mother and child cohort study (INMA: Infancia y Medio Ambiente / Childhood and Environment; www.proyectoinma.org) 40 cord blood samples with known concentrations of mercury and seven organochlorines, including PCBs, DDTs, hexachlorobenzene (HCB), and beta-hexachlorocyclohexane (β -HCH) have been analyzed for their content in two excitatory amino acids, glutamate and aspartate. The associations between these amino acids and the OCs and mercury have been analyzed. Binding assays with human placental homogenates exposed to methylmercury and OCs have also been performed.

2. Material and methods

2.1. Population and sample collection

Population

Informed consent was obtained from all participants and the study was approved by the Hospital La Fe (Valencia, Spain) Ethics Committee. The study population included 40 mother-child pairs among the 787 deliveries of the INMA-Valencia cohort (Guxens et al. 2012). Selection criteria of the samples were based on availability of cord blood and placenta samples for the determinations as well as on total mercury (T-Hg) concentrations. Thus, the consecutive 20 children with the highest levels of exposure to T-Hg and the successive 20 with the lowest levels were selected. All children in the highest group had levels of mercury >20 µg/L and, in the case of the group of lower levels, all children had <6.6 µg/L of mercury. The selected samples also had cord blood analyses of some OCs, including HCB, β-HCH, 4,4'-DDT, 4,4'-DDE, and PCBs 138, 153 and 180. Total cholesterol and triglycerides were determined by means of enzymatic techniques, calculating total serum lipid concentrations (Phillips et al., 1989). The pollutant levels of the INMA-Valencia cohort, the laboratory analytical methods and quality control procedures are described elsewhere (Lopez-Espinosa et al., 2010; Ramon et al., 2008, 2011; Vizcaino et al., 2010, 2011). Levels of these contaminants (n=40) have been described in Table 1.

Umbilical cord and placenta samples

Umbilical vein blood samples were collected using venipuncture of cord vessels before the placenta was delivered. Serum and plasma samples were obtained after whole blood centrifugation at 2500 rpm and separated into aliquots of 1 mL. Placentas collected at delivery were examined, and pieces of maternal and fetal sides were immediately dissected. Both cord and placenta samples were coded, frozen and stored confidentially and anonymously at -80 °C until processed.

2.2. Chemicals

Saccharose, Tris-HEPES, glacial acetic acid and protease inhibitor cocktail (P8340) were purchased from Sigma (St. Louis, USA). Chemical standards, methylmercuric chloride, PCB 138 and β -HCH were from ICN (Cleveland, OH, USA), Dr. Ehrenstoner GmbH (Augsburg, Germany), and National Physical Laboratories UK (Teddington, United Kingdom), respectively. [^3H]-D-aspartate (27 Ci/mmol) was from Amersham Life Sciences (Buckinghamshire, UK). Optiphase 'Hisafe' 2 liquid scintillation cocktail was from Wallace Oy (Turku, Finland). The chemicals were dissolved and diluted in Tris-acetate buffered solution or in dimethyl sulfoxide solution (DMSO). When dissolved in DMSO, a 200 $\times$ concentration was prepared, thus the concentration of DMSO in the testing solution was 0.5%. Controls contained the same amount of DMSO, when so required.

L-glutamic acid, L-aspartic and *o*-phthalaldehyde/-mercaptoethanol (50:50) reagent solutions were from Sigma (St. Louis, USA). Ortho-phosphoric acid was from Merck. All other chemicals were from Sigma (St. Louis) and Merck (Darmstadt, Germany).

Primary antibodies goat polyclonal for the rat excitatory amino acid transporter 2 (EAAT2) (1:1000) and mouse monoclonal anti- Na^+/K^+ -ATPase α 1 (1:200) were from BD Biosciences and Santa Cruz Biotechnology, Inc, respectively. Secondary anti-goat horseradish peroxidase-conjugated (HRP) and HRP linked anti-mouse (1:1500) were from Molecular Probes.

2.3. Amino acid determination

Glutamate and aspartate cord plasma concentrations were determined by high performance liquid chromatography (HPLC)-fluorimetric analysis (Waters, Milford, MA; and Applied Biosystems, Foster City, CA) (Lopez-Gil et al., 2007; Reynolds et al., 2002). In brief, plasma samples were thawed and an aliquot of 25 μL was mixed with 475 μL of 0.14 M perchloric

acid. After 10 minutes in an ice bath, the sample was centrifuged during 2 minutes at 14000xg and 4°C (x2). The supernatant was filtered through millex®-HV filters (0.45 µm) and divided in aliquots that were frozen at -80°C until the analysis. Amino acids were derivatized with o-phthalaldehyde/-mercaptoethanol reagent solution (50:50) for 5 minutes and injected into a reverse-phase C18 column (Tracer Nucleosil C18 5-µm particle size, 10 x 0.4 cm; Teknokroma, Spain). The separation of the amino acids was performed using a mobile phase gradient consisting of 100 mM sodium phosphate dissolved in mili-Q water adjusted to pH 6.4 with ortho-phosphoric acid (Merck) and 28% methanol, followed by a lineal gradient up to 80 % methanol in water for 4 minutes. The system was kept on 80% methanol in water for 2 minutes and went back to the initial conditions in 5 minutes using a lineal gradient. Mobile phase flow was 0.8 ml/min. Both mobile phases were continuously degassed with helium. Samples were injected every 20 minutes. The amount of glutamate and aspartate was quantified by fluorescence detection (Excitation at 360nm; Emission at 450nm) with an external standard method using L-glutamic acid and L-aspartic acid dissolved in 0.14 M perchloric acid. A 9 point standard curve was run with each series of HPLC analysis and linearity was assured ($r^2 > 0.99$). The forty plasma samples have been analyzed in triplicate-quintuplicate, in different assays during a two month period. A control solution of 1 µM glutamate was analyzed every each 10 samples to confirm that assay parameters remained constant. The mean of the intra-assay coefficient of variation (CV) was 5.5% for 8 experiments, and the inter-assay CV was 9.1%.

2.4. Placental plasma membrane homogenates preparation

The placental plasma membrane homogenates were prepared following a described protocol (Jimenez et al., 2004) with some modifications. Firstly, fetal placental pieces of the fetal face were thawed, and the amount needed was taken (~10 g was used for each assay). Placental

tissue was chopped into small pieces, washed with 0.9 % NaCl solution to remove blood and filtered through gauze. Then, the tissue was homogenized eight times (30 sec each time) at low speed with a B. Braun Potter S homogenizer in three volumes of ice-cold buffer A (250 mM saccharose, 10 mM Tris-HEPES adjusted at pH 7.4 with glacial acetic acid) and a protease inhibitor cocktail (1:200) was added after homogenization. The homogenate was centrifuged at 1000xg for 10 min and the supernatant was removed and retained. The pellet was resuspended in buffer A and homogenized for 30 sec five times. This second homogenate was centrifuged again at 1000xg for 10 min and the supernatants from both centrifugations were combined. The supernatant was then centrifuged at 10 000xg for 15 min. The pellet corresponding to the mitochondria was discarded and the supernatant was centrifuged at 100000xg for 1h 20 min with an L-90K ultracentrifuge (Beckman Coulter Optime™); the pellet was resuspended in 2 mL of buffer A. After that, protein content was determined.

2.5. Glutamate transport

Methylmercury, PCB138 and β -HCH were tested against glutamate transport in placenta based in the following facts: i) methylmercury is the most neurotoxicant form of mercury and accounts for $\geq 90\%$ of total mercury in cord blood in populations with high levels of exposure (Mahaffey 2005) and ii) PCB138 and β -HCH levels were associated with glutamate levels in cord blood (see Results section).

Glutamate transporter EAAT2 expression was determined by Western Blot following current protocols as the one described elsewhere (Briz et al., 2010; Fonfría et al., 2005). 20 μ l of placental homogenates containing 5.4 μ g of protein were used. They were boiled during 5 minutes and then were subjected to SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide resolving gel at 100 mA for 1.5h-2h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% non-fat dry milk in Tris-Buffered Saline

Tween-20 (TBS-T) (20 mM Tris-HCl [pH 7.6], 140 mM NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4°C with a goat polyclonal anti-rat EAAT2 (1:1000; BD Biosciences) in TBS-T solution containing 5% bovine serum albumin (BSA). After membrane washing, they were incubated with anti-goat horseradish peroxidase–conjugated (HRP) secondary antibodies (1:1500; Molecular Probes). A monoclonal anti-Na⁺/K⁺-ATPase α 1 (1:200; Santa Cruz Biotechnology, Inc.) and a secondary HRP linked anti-mouse (1:1500) antibodies were used to control for the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP kit; Bio-Rad, Hercules, CA). Luminescence was quantified with a Versadoc Imagine System (Bio-Rad). Digital images were then quantified in the Quantity One software (Bio-Rad). Glutamate transport was determined as previously described (Fonfría et al., 2005; Galofré et al., 2010) as [³H]-D-aspartate uptake, since D-aspartate is a non-metabolizable glutamate analogue. The binding of [³H]-D-aspartate to EAAT transporters in human placenta has been performed following the method described for human brain frozen tissue and platelets (Borges et al., 2007; Cross et al., 1986) with some modifications. In brief, placental tissue homogenate containing 150 µg protein was incubated in 1mL reaction mixture containing 50 mM Tris-acetate buffer (pH=7.4), 200 mM NaCl, and 20 nM [³H]-D-aspartate (1 TBq/mmol) in presence or absence of methylmercury, PCB138, β -HCH and glutamate, at different concentrations. Incubation was carried out at 25°C for 1 hour. Reaction was stopped by rapid vacuum filtration onto Whatman GF/C fibers filters using Brandell Cell Harvester equipped with 24-well probe. Filter sheets were soaked in ice-cold incubation buffer prior to use. Each individual filter was placed in 3ml vial scintillation vials with 2mL of scintillation cocktail; the activity in each vial was determined by liquid scintillation spectrometry.

2.6. Statistical analysis

Descriptive analysis included geometric means, 95% confidence intervals (CIs), and percentiles, for contaminants and outcomes. Response and exposure variables were right-skewed, thus they were log₁₀-transformed to approach normality. The association of glutamate and aspartate with contaminants was analyzed using linear regression analyses adjusting by serum lipids. Organochlorine compounds with detection frequency < 50% were analyzed as a dichotomous variable (below or above the limit of detection –LOD–), while mercury was analyzed both as a continuous and a dichotomous variable (low versus high mercury concentrations). Values below LOD were imputed LOD/sqrt(2) so correlations include this data (CDC, 2009). Due to high differences in contaminant concentration between Spanish and Latin American (n=6) women (Table 1), main analyses were performed excluding women from Latin America. Results for the whole population are shown in the Supplementary material. Interaction between OCs and T-Hg on glutamate was also assessed by including the product term between T-Hg (in two categories: low versus high level of exposure) and OCs (in logarithmic scale). All analyses were performed with SPSS statistical software package version 17 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

Results of [³H]-D-aspartate binding assays are expressed as mean ± SD. Concentration-response curves from two to three independent experiments, each one performed using triplicates for each concentration point, were fitted to sigmoid curves using the GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). At least, 6 concentrations of each pollutant were used to define the curve.

3. Results

3.1. Concentrations of contaminants and amino acids in cord samples

Table 1 shows the concentrations of T-Hg and OCs detected in the cord samples expressed in

ng/mL. Briefly, median concentrations of T-Hg, HCB, β -HCH, 4,4'-DDE, and Σ PCBs were 13.8, 0.29, 0.13, 0.54, 0.36 ng/mL, respectively. Latin American women had significantly lower levels of mercury, HCB and PCBs, and higher levels of DDE as it is showed in table 1. Due to these remarkable differences, we decided to exclude these women from the main analyses (Tables 2 - 4). Information including the whole sample size is shown in Supplementary Material.

Table 1 also shows the concentrations of glutamate and aspartate levels (medians: 35 and 5.3 μ mol/L) in cord plasma. They were positively correlated (Pearson $r = 0.75$, $p < 0.001$) (See Supplemental Material, Figure 1).

3.2. Association between amino acid levels in human umbilical plasma and toxic compounds.

The relationships between glutamate and aspartate and pollutant levels in cord blood when excluding Latin-American women ($n=34$) are shown in Tables 2-3. We found a significant negative relationship between glutamate and the sum of the three PCBs, and the seven OCs in cord blood after adjusting for lipids ($p = 0.02$ in both cases) (Table 2). More specifically, β -HCH and PCB138 showed a significant negative relationship with glutamate, with a decrease of 24 and 51% (p -values of 0.04 and 0.02, respectively) in glutamate levels for each 10-fold increase in the contaminant concentration. HCB, 4,4'-DDE and PCB180 showed a negative trend, although they did not reach statistical significance. On the other hand, no significant relationship was found between T-Hg concentration and the levels of glutamate in the blood samples. Finally, we did not find any significant relationship for aspartate and the pollutants analyzed in our samples (Table 3). Patterns were similar in the analyses including the whole sample ($n=40$), but, only the sum of the 7 OCs remained statistically significant (See Supplemental material, Table 1-2).

We analyzed if the association between OCs and glutamate levels varied according to T-Hg exposure. Samples were stratified in two models consisting of low ($< 6.6 \mu\text{g/L}$) and high ($> 20 \mu\text{g/L}$) T-Hg exposure. Table 4 shows the association between OCs and glutamate levels stratified by T-Hg exposure. There was an interaction pattern ($p < 0.10$) in the case of 4,4'-DDE or PCB138, with a significant inverse association between glutamate and 4,4'-DDE or PCB138 (coefficients: -73 and $-75 \mu\text{mol/L}$, respectively; $p = 0.01$) with low T-Hg concentrations and a lack of relation at high T-Hg concentrations (Figure 1).

3.3. Glutamate transport in human placenta is inhibited by environmental contaminants

We analyzed placental plasma membrane homogenates for the expression of EAAT2 in order to assure the presence of glutamate transporter in our placental samples (Figure 2A). [^3H]-D-aspartate binding in placental homogenates was inhibited by glutamate with a 50% inhibitory concentration (IC₅₀) of $6.3 \pm 1.7 \mu\text{M}$ (Figure 2B).

We tested the effects of methylmercury and of PCB138 and β -HCH, since the latests were associated with levels of glutamate, on glutamate transport in placenta. Methylmercury completely inhibited [^3H]-D-aspartate binding in placental homogenates with 20% and 50% inhibitory concentration (IC₂₀ and IC₅₀ respectively) values of $2.1 \pm 1.0 \mu\text{M}$ and $4.9 \pm 0.8 \mu\text{M}$, respectively (Figure 3). PCB 138 and β -HCH partially inhibited [^3H]-D-aspartate binding with IC₅₀ values of $14.2 \pm 1.2 \text{ nM}$ and $6.9 \pm 2.9 \text{ nM}$, respectively. Despite the high affinity of PCB138 and β -HCH for the glutamate transport they did not completely inhibit it. The maximum inhibitory effects observed were $46 \pm 14 \%$ and $55 \pm 1 \%$ for PCB138 and β -HCH, respectively, and they were attained at concentrations $\geq 1 \mu\text{M}$ (Figure 3).

4. Discussion

The present work examines the effect of some organic pollutants in glutamatergic parameters of human samples related to the development (umbilical cord blood and placenta). The 40 umbilical cord blood samples chosen for this work had comparable levels of pollutants than the whole cohort of Valencia where they belong: the T-Hg geometric mean was 9.2 ng/L here while it was 9.9 ng/L in the whole cohort (Ramon et al. 2008). At the same time the OCs compounds of this subgroup had similar levels to those of the whole cohort (Vizcaino et al. 2011). The group of Latin American women was excluded from the study since the mother's country origin appears to be an important determinant for patterns of mercury and OC concentrations in this group and also in the whole cohort (table 1; Llop et al., 2010; Ramon et al., 2011), which is also relevant for the concentrations of these pollutants in-utero (Vizcaino et al., 2010). If we take into account the possible interaction between the contaminants, the presence of two different groups in the studied population may distort the output result.

The values of glutamate and aspartate and their relationship in human cord blood determined here (35 and 5 $\mu\text{mol/L}$, respectively) were in the range of previously reported data (19 to 285 $\mu\text{mol/L}$ for glutamate and 7 – 10 times lower for aspartate) (Bajoria et al., 2001; Cetin et al., 2005; Jauniaux et al., 2001 and Marconi et al., 2010). We found a significant negative correlation between the glutamate concentration in cord blood and the levels of PCB138 and β -HCH, once adjusting for lipid content and excluding Latin American women from the study. Finally, the sum of the three PCBs and all OCs also decreased significantly the levels of glutamate. With regards to mercury we did not find an association with glutamate levels. At the same time, no relationship was found between aspartate and the pollutants levels.

In this study we have considered that the real scenery is that population is exposed to several pollutants at the same time, which may have compensatory, additive or synergic effects. Therefore, we wonder whether the interaction between pollutants could affect the output of the present results. Our results showed that PCB138, and 4,4'-DDE were inversely associated

with glutamate for the samples with low T-Hg concentrations (6.6 µg/L) in cord blood, but not for the samples with high T-Hg concentrations, suggesting compensatory effects between OCs and mercury. In this way, there are experimental evidences that methylmercury counteract with PCBs when supplied in complex mixtures. Thus, it has been reported that methylmercury attenuated hearing and learning deficits induced by exposure to PCBs in developing rats (Piedrafita et al., 2008; Powers et al., 2009).

We also showed that human placenta expressed glutamate transporters by determining both the EAAT2 protein expression and the inhibition of [³H]-D-aspartate binding by the amino acid glutamate. We have determined the protein expression of EAAT2 in our placental samples because it manages 90% of the glutamate uptake and it is found both in the syncytiotrophoblast layer of the placenta and in endothelial cells of fetal blood vessels (Danbolt, 2001; Noorlander et al., 2004). To the best of our knowledge, we demonstrated for the first time that methylmercury completely inhibits glutamate transport in human placental tissue, like it does in platelets and neural cells (Aschner et al., 2000; Borges et al., 2007; Fonfría et al., 2005). The affinity of methylmercury for EAAT transporters, as measured by its IC₅₀ value against [³H]-aspartate uptake, is similar in placenta (4.9 µM, this work) and in both brain synaptic membranes (4.7 µM, Porciúncula et al., 2003) and living neurons in culture (12 µM, Fonfría et al., 2005). Placental EAAT are involved in active transport of glutamate between the fetal and maternal blood circulation, regulating the clearance of glutamate from the fetal circulatory system (Battaglia, 2000; Noorlander et al., 2004). Therefore an inhibition of placental glutamate transport could impair the absorption of glutamate by the placenta. Although there is no much knowledge about the physiological effects of an inhibition of the EAAT in the placenta, we speculate that this might be deleterious for the fetal wellbeing and development. Due to the inhibition by methylmercury of EAATs in the placenta, we expected to find an increase of the glutamate concentration in

umbilical cord blood in relation to these pollutants. The lack of a significant association for Hg and glutamate or aspartate levels could be due to the fact that the highest levels of T-Hg found in the samples ($66 \mu\text{g/L} \equiv 0.33 \mu\text{M}$) are lower than the concentrations of methylmercury that significantly inhibit [^3H]-aspartate uptake (IC₂₀ value: $2.1 \mu\text{M}$). It should be noticed that mercury levels in umbilical cord blood and placenta are positively correlated (Kozikowska et al., 2013; Ask et al., 2002). We also found that PCB 138 and β -HCH inhibited glutamate transport in human placentas with high affinity ($\sim 10 \text{ nM}$) but low efficacy ($\sim 50 \%$ of maximum inhibition). The findings with PCB 138 are in agreement with those reporting that other higher chlorinated ortho-PCBs partially inhibit the uptake of glutamate in rat brain synaptosomes (Stavenes Andersen et al., 2009; Mariussen and Fonnum, 2001). Also, PCB 138 impairs glutamate-related pathways in cultured cerebellar neurons with a high potency (Llansola et al., 2010). With respect to β -HCH, as far as we know, there are no reports on its effect on glutamate transport.

With regards to PCB138 and β -HCH, their maximum concentrations in cord blood (1.1 and 4.2 nM, respectively) were similar to their IC₅₀ values against glutamate uptake in human placenta. However, since these two compounds did not produce a complete inhibition of the glutamate transport, there would be still enough glutamate transport activity to avoid an increase of extracellular glutamate. In addition to the transport, other factors might contribute to alter the glutamate levels in the cord blood, like disrupted synthesis/degradation of glutamate in the fetal liver or transamination of branched chain amino acids to provide glutamate in the placenta (Neu , 2001). In this way, a proteomic study revealed a reduction in the glutamate synthesizing enzyme glutaminase after amphipod chronic exposure to PCB 77 and 169 (Leroy et al., 2010). However, to the best of our knowledge, there are no reports on whether PCB138 or β -HCH interacts with glutamate metabolic pathways.

The developing fetal compared to adult brain is more vulnerable to the effects of toxic agents

because of differential exposure, physiologic immaturity, and a longer lifetime over which disease initiated in early life can develop (Castoldi et al., 2008; Grandjean and Landrigan, 2006; Perera et al., 1999). It also shows different influx of amino acids either due to a deficient blood brain barrier or to increased transport mechanisms (Saunders et al., 2012 and references herein). These factors may result in subtle changes in brain glutamate concentration related to environmental toxicant exposure, which eventually could be associated with neurological deficits observed in exposed children (Grandjean and Landrigan, 2006; Sunyer et al., 2010). In this sense, reduced levels of glutamate/glutamine in several brain areas have been found in adults with childhood lead exposure (Cecil et al., 2010). Regarding our work, recent studies performed on children up to 2 years of age participating in the INMA Project found an inverse association between PCB 138 concentrations and impairment of psychomotor development (Forns et al. 2012a). This would be in agreement with the negative association between glutamate and PCB 138 levels in cord blood found in this work. It should be stressed that glutamate exerts a crucial role on motor coordination through the activation of metabotropic glutamate receptors (Nakao et al., 2007). In addition, Gascon et al. (2013) highlighted the negative role of prenatal exposure to PCB in comparison to postnatal exposure including breast-feeding. However, no association was found between mercury concentrations and mental or psychomotor development in two-years old children in the INMA cohort (Llop et al., 2012). Other studies reported adverse effects of mercury on visual responses and linguistic pathways, however levels of exposure were higher than the ones reported in the present work (Ramirez et al., 2003; Yorifuji et al., 2013).

In conclusion, we showed that the levels of glutamate in umbilical cord blood were inversely associated with PCB138 and β -HCH concentrations, and to the sum of three PCBs and all OCs. A compensatory effect between PCB138 and 4,4'-DDE with mercury was found in accordance with previous animal experiments. We also found that methylmercury, PCB138

and β -HCH inhibit glutamate transport in human placentas. The affinity of these compounds against [3 H]-D-aspartate binding was β -HCH $\geq$ PCB 138 > methylmercury, however complete inhibition was only attained by the latest. These preliminary results may contribute to the understanding of the effects of exposition to environmental levels of OCs and mercury compounds in human neurodevelopment. Further research involving higher numbers of participants should be implemented for further insight into these interactions which is needed in view of the lack of previous studies on this issue.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version

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FIGURES LEGENDS

Figure 1. Linear correlation of 4,4'-DDE (A) and PCB138 (B) levels with glutamate concentration in human cord blood in the samples containing low levels of T-Hg ($< 6.6 \mu\text{g/L}$) (○, solid line) in comparison to the lack of association in the samples containing high levels of T-Hg ($> 20 \mu\text{g/L}$) (*, dashed line).

Figure 2. A) Representative immunoblot for the glutamate transporter EAAT2 in human placental membranes homogenates, and the corresponding immunoblot for Na^+/K^+ -ATPase $\alpha 1$ as a membrane marker. Molecular weights are given on the left. **B)** Concentration-dependent inhibition of [^3H]-D-aspartate in human placental membranes homogenates by glutamate. The data correspond to mean $\pm$ SE of three independent experiments, each determined in triplicate.

Figure 3. Inhibition of [^3H]-aspartate binding on human placental plasma membrane by methylmercury (▼—), PCB138 (●---) and β -HCH ($\Delta \cdots$). Values are mean $\pm$ SD of 2-3 experiments, each performed in triplicate.

A **B**

A **B**

TABLES

Table 1. Descriptive statistics of the pollutants and amino acids analyzed in cord blood . INMA-Valencia cohort (Spain), 2004-2006

	LOD	Whole sample (n=40)											Spanish (n=34)			Latin American (n=6)		
		%≥LOD	GM	(95% CI)		P 25	P 50	P 75	P 25	P 50	P 75	P 25	P 50	P 75				
Pollutants (ng/mL)																		
T-Hg	2.0	65.0	9.2	(5.5;	15.4)	1.4	13.8	42.8	1.4	32.5	43.3	1.4	1.4	5.7	*			
4,4'-DDT	0.009	47.5	-	-	-	<LOD	<LOD	0.07	<LOD	<LOD	0.06	<LOD	0.03	0.08				
4,4'-DDE	0.016	100.0	0.51	(0.41;	0.64)	0.30	0.54	0.80	0.28	0.48	0.76	0.55	1.13	1.46	*			
HCB	0.035	92.5	0.21	(0.14;	0.30)	0.12	0.29	0.49	0.14	0.34	0.58	0.03	0.11	0.24	*			
β-HCH	0.008	82.5	0.07	(0.04;	0.11)	0.05	0.13	0.16	0.06	0.14	0.17	0.03	0.07	0.12				
PCB 138	0.031	90.0	0.08	(0.06;	0.10)	0.04	0.10	0.13	0.06	0.10	0.13	0.02	0.02	0.16	*			
PCB 153	0.020	95.0	0.10	(0.08;	0.13)	0.06	0.12	0.19	0.09	0.16	0.20	0.01	0.04	0.07	*			
PCB 180	0.018	95.0	0.07	(0.05;	0.09)	0.04	0.09	0.12	0.06	0.10	0.13	0.01	0.02	0.04	*			
Σ ₃ PCBs	-	-	0.27	(0.21;	0.34)	0.20	0.36	0.46	0.23	0.39	0.46	0.05	0.10	0.29	*			
Σ ₇ OCs	-	-	1.30	(1.08;	1.55)	1.00	1.47	1.82	0.98	1.54	1.85	1.12	1.40	1.64				
Amino acids (μmol/L)																		
Glutamate	1	100	38.0	(32.0;	45.0)	26.8	35.3	55.6	26.6	33.9	54.9	26.7	56.2	74.0				
Aspartate	1	100	6.8	(5.3;	8.6)	3.9	5.3	11.2	3.7	4.9	9.0	5.3	15.1	31.9				

CI: Confidence interval; GM: Geometric mean; HCB: Hexachlorobenzene; HCH: Hexachlorocyclohexane; LOD: Limit of detection; PCBs: Polychlorobiphenyls; P:

Percentile; Σ₃PCBs: Sum of three PCBs (PCB 138, 153, and 180) ; Σ₇OCs: Sum of the seven organochlorine compounds; T-Hg: Total Mercury.

* p<0.05, p-value from Mann-Whitney test comparing pollutants and amino acids concentrations according to origin

Table 2. Association between glutamate and pollutant levels in cord blood^{a,b}

Pollutants (ng/mL)	n	Unadjusted models			Adjusted for lipids ^c		
		%change ^d	(95% CI)	p	%change ^d	(95% CI)	p
log ₁₀ T-Hg	34	8	(-18; 42)	0.56	4	(-20; 35)	0.77
T-Hg<6.6	14	Ref			Ref		
T-Hg>20	20	13	(-23; 66)	0.53	6	(-26; 53)	0.74
4,4'-DDT<LOD	18	Ref			Ref		
4,4'-DDT>LOD	16	-26	(-48; 7)	0.11	-16	(-42; 23)	0.37
log ₁₀ 4,4'-DDE	34	-36	(-66; 23)	0.17	-41	(-68; 7)	0.08
log ₁₀ HCB	34	-28	(-51; 6)	0.09	-29	(-50; 2)	0.06
log ₁₀ β-HCH	34	-18	(-37; 8)	0.16	-24	(-41; -2)	0.04
log ₁₀ PCB138	34	-39	(-69; 19)	0.14	-51	(-73; -10)	0.02
log ₁₀ PCB153	34	-27	(-58; 28)	0.26	-33	(-60; 13)	0.13
log ₁₀ PCB180	34	-41	(-72; 22)	0.15	-48	(-73; 2)	0.06
log ₁₀ (Σ ₃ PCBs)	34	-50	(-76; 5)	0.06	-56	(-77; -13)	0.02
log ₁₀ (Σ ₇ OCs)	34	-48	(-74; 3)	0.06	-54	(-76; -14)	0.02

CI: Confidence interval; HCB: Hexachlorobenzene; HCH: Hexachlorocyclohexane; LOD: Limit of detection; PCBs: Polychlorobiphenyls; P: Percentile; Ref: reference category; Σ₃PCBs: Sum of three PCBs (PCB 138, 153, and 180) ; Σ₇OCs: Sum of the seven organochlorine compounds; T-Hg: Total Mercury.

^a Latin American women were excluded from analysis (n=6), because of remarkable differences in mercury and OC concentrations from the Spanish ones

^b Linear regression models between glutamate and pollutants, where all variables were log₁₀-transformed.

^c Lipids were included as a separate term in the models.

^d Coefficients were exponentiated ($(10^{\text{coef}}-1)*100$) and interpreted as the percentage change in glutamate by every 10-fold increase in the pollutant level.

Table 3. Association between aspartate and pollutant levels in cord blood^{a,b}

Pollutants (ng/mL)	n	Unadjusted models			Adjusted for lipids ^c		
		%change ^d	(95% CI)	p	%change ^d	(95% CI)	p
log ₁₀ T-Hg	34	13	(-20; 59)	0.47	10	(-22; 55)	0.57
T-Hg<6.6	14	Ref			Ref		
T-Hg>20	20	31	(-18; 110)	0.25	26	(-22; 103)	0.33
4,4'-DDT<LOD	18	Ref			Ref		
4,4'-DDT>LOD	16	-35	(-59; 2)	0.06	-31	(-58; 12)	0.12
log ₁₀ 4,4'-DDE	34	-28	(-68; 64)	0.43	-32	(-70; 53)	0.34
log ₁₀ HCB	34	-22	(-53; 28)	0.32	-23	(-53; 27)	0.30
log ₁₀ β-HCH	34	-19	(-42; 14)	0.22	-23	(-46; 8)	0.13
log ₁₀ PCB138	34	-30	(-70; 63)	0.40	-40	(-75; 41)	0.23
log ₁₀ PCB153	34	-14	(-57; 75)	0.67	-19	(-60; 64)	0.55
log ₁₀ PCB180	34	-45	(-78; 37)	0.19	-50	(-80; 24)	0.13
log ₁₀ (Σ ₃ PCBs)	34	-39	(-77; 59)	0.30	-44	(-78; 45)	0.22
log ₁₀ (Σ ₇ OCs)	34	-47	(-78; 30)	0.16	-51	(-80; 18)	0.11

CI: Confidence interval; HCB: Hexachlorobenzene; HCH: Hexachlorocyclohexane; LOD: Limit of detection; PCBs: Polychlorobiphenyls; P: Percentile; Ref: reference category; Σ₃PCBs: Sum of three PCBs (PCB 138, 153, and 180) ; Σ₇OCs: Sum of the seven organochlorine compounds; T-Hg: Total Mercury.

^a Latin American women were excluded from analysis (n=6), because of remarkable differences in mercury and OC concentrations from the Spanish ones.

^b Linear regression models between aspartate and pollutants, where all variables were log₁₀-transformed.

^c Lipids were included as a separate term in the models.

^d Coefficients were exponentiated ((10^{coef}-1)*100) and interpreted as the percentage change in aspartate by every 10-fold increase in the pollutant concentration

Table 4. Association between glutamate and OCs at high (>20 µg/L) or low (<6.6 µg/L) levels of T-Hg^{a,b}

Pollutants (ng/mL)	Unadjusted models				Adjusted for lipids ^c			
	%change ^d	(95% CI)	p	p(inter)	%change ^d	(95% CI)	p	p(inter)
4,4'-DDT>LOD				0.16				0.16
Model 1 (T-Hg<6.6)	0	(-52; 109)	1.00		29	(-56; 276)	0.61	
Model 2 (T-Hg>20)	-40	(-60; -11)	0.02		-33	(-55; 0)	0.05	
log ₁₀ 4,4'-DDE				0.12				0.05
Model 1 (T-Hg<6.6)	-65	(-87; -8)	0.04		-73	(-90; -29)	0.01	
Model 2 (T-Hg>20)	-2	(-64; 169)	0.97		0	(-59; 145)	1.00	
log ₁₀ HCB				1.00				0.92
Model 1 (T-Hg<6.6)	-44	(-69; 1)	0.05		-43	(-69; 6)	0.07	
Model 2 (T-Hg>20)	-44	(-77; 37)	0.19		-39	(-72; 34)	0.20	
log ₁₀ β-HCH				0.75				0.44
Model 1 (T-Hg<6.6)	-30	(-54; 6)	0.09		-34	(-57; 2)	0.06	
Model 2 (T-Hg>20)	-39	(-71; 30)	0.19		-49	(-73; -5)	0.03	
log ₁₀ PCB138				0.06				0.07
Model 1 (T-Hg<6.6)	-70	(-88; -25)	0.01		-75	(-90; -40)	0.01	
Model 2 (T-Hg>20)	14	(-63; 249)	0.81		-12	(-68; 143)	0.80	
log ₁₀ PCB153				0.91				0.74
Model 1 (T-Hg<6.6)	-51	(-80; 20)	0.11		-56	(-82; 12)	0.08	
Model 2 (T-Hg>20)	-45	(-88; 147)	0.41		-40	(-84; 127)	0.43	
log ₁₀ PCB180				0.21				0.54
Model 1 (T-Hg<6.6)	-60	(-85; 6)	0.06		-60	(-86; 17)	0.09	
Model 2 (T-Hg>20)	10	(-71; 325)	0.88		-33	(-81; 132)	0.50	
log ₁₀ (Σ ₃ PCBs)				0.52				0.65
Model 1 (T-Hg<6.6)	-71	(-89; -23)	0.02		-71	(-89; -21)	0.02	
Model 2 (T-Hg>20)	-42	(-92; 325)	0.57		-55	(-92; 156)	0.35	
log ₁₀ (Σ ₇ OCs)				0.26				0.23
Model 1 (T-Hg<6.6)	-76	(-91; -35)	0.01		-77	(-91; -39)	0.01	
Model 2 (T-Hg>20)	-39	(-85; 145)	0.46		-44	(-84; 88)	0.33	

CI: Confidence interval; LOD: Limit of detection; HCB: Hexachlorobenzene; HCH: Hexachlorocyclohexane; PCBs: Polychlorobiphenyls; p(inter): P-value for the interaction between T-Hg and the OCs; Σ₃PCBs: Sum of three PCBs (PCB 138, 153, and 180); Σ₇OCs: Sum of the seven organochlorine compounds; T-Hg: Total Mercury.

^a Latin American women were excluded from analysis (n=6), because of remarkable differences in mercury and OC concentrations from the Spanish ones.

^b Linear regression models between glutamate and OCs, where all variables were log₁₀-transformed.

^c Lipids were included as a separate term in the models.

^d Coefficients were exponentiated ((10^{coef}-1)*100) and interpreted as the percentage change in glutamate by every 10-fold increase in the pollutant level.