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NEURODEVELOPMENTAL TOXICITY OF POLYBROMINATED DIPHENYL ETHERS (PBDE) AT DOCUMENTED HUMAN EXPOSURE LEVELS IN A MOUSE MODEL OF PRENATAL EXPOSURE.

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Introduction

Neurobehavioral disorders and brain dysfunctions have been reported in children exposed in utero to a variety of industrial chemicals, even if present in the environment at minimal sub-toxic concentrations [1].

Polybrominated diphenyl ethers (PBDE) are persistent and ubiquitous organic contaminants commonly used as flame retardants. In contrast with other contaminants like dioxins and PCBs, PBDE levels have been reportedly increasing in the environment and in human tissues in the latest years. Epidemiological studies suggest a correlation between maternal exposure to PBDE and signs of impairment in fetal development [2-3]. The identification of potential neurotoxic effects and mechanisms of PBDE in the developing mammal central nervous system (CNS) are thus of primary interest in order to define a risk characterization for humans.

We present here evidence of neurotoxicity by realistic decaBDE doses (reproducing the levels of human exposure) on fetal neurodevelopment in mice. Methylmercury was used as reference neurotoxic contaminant, and to evaluate possible synergism with decaBDE. DecaBDE- and MeHg-dependent alterations were evaluated in developing neurons in mouse primary cultures, and in a mouse model of prenatal exposure to the toxicants.

Materials and Methods

Cell Cultures and In Vitro Analysis

Primary neuron and glia cultures were obtained from 13-day-old (E13) C57BL/6J mouse embryos by adapting different protocols [4, 5]. Neurodevelopmental alterations were studied by immunocytochemistry in hippocampal and cerebellar neuron/glia cocultures [6]. Cultures were exposed to the contaminants for 72 hours, and Z-stack pictures of immunostained cells were acquired with a laser scanning microscope. Deconvolution, three-dimensional (3D) reconstructions and data analysis were then performed. Dendritic arborization, pre-/post-synaptic proteins (synaptophysin and PSD95), and a marker of immature neurofilaments (doublecortin, DCX) were analyzed. To determine the activation of immunocompetent cells, the morphological parameters (cell area and circularity) of cultured microglia were measured with ImageJ v1.43 software (National Institutes of Health). The compounds tested were DE-83R (a decabromodiphenyl ether mixture, Wellington Laboratories) and methylmercury chloride (MeHg, Sigma Aldrich).

Animal Model and Ex Vivo Analysis

Pregnant mice were randomly assigned to the different experimental groups and orally treated once a day from gestational day 6 to 9 by oral gavage. Brains were explanted from E13 embryos or newborn mice. The quantification of DCX, synaptophysin, PSD95, CD68 and GFAP was performed by Western blot on brain samples. The expression of the calcium-binding protein Iba-1, GFAP, doublecortin, and synaptophysin in hippocampus and cerebellum was analyzed on serial paraffinized brain slices from newborn mice by immunohistochemistry.

Results and discussion

Non lethal concentrations of decaBDE impair neurodevelopmental markers in primary neuron cultures and in the brain of in utero-exposed fetuses.

The effects of non-lethal concentrations of decaBDE and MeHg on synaptic and neurofilament markers were analyzed in neuron/glia cocultures. Dendritic arborization of treated cultured neurons was analyzed by automated assessment of the number of dendritic branches and Sholl intersections (ramification index) on 3D reconstructed branching. A significant decrease in dendritic branches and Sholl intersections (about 40% vs. control; Fig 1A, B) was induced by DE-83R down by 0.42 nM. To detect possible alterations in neuron maturation, cultures were immunostained with specific antibodies for immature (DCX) and mature (NF200) neurofilaments. In hippocampal neurons, DE-83R significantly increased the DCX/NF200 ratio down by 0.42 nM (Fig 2). MeHg did not induce any significant effect, and not further increase was observed after combined treatments. Semi-quantification of synaptophysin and

PSD95 signals revealed that exposure to DE-83R or MeHg decreased the expression of both the synaptic proteins in hippocampal neurons upwardly by the lowest tested concentrations (Fig 3). Synaptic protein expression was not altered by the contaminants in cerebellar neuron cultures (not shown).
Ex vivo analysis were performed on brains from mouse embryos prenatally exposed to DE-83R/MeHg in order to verify the effects of the contaminants in vivo. Synaptophysin expression was reduced by about 64÷76% by 0.3 up to 30 mg/kg bw DE-83R (Fig 4A). PSD95 exhibited a similar trend towards a decreased expression (by 28÷40%, from 0.3 up to 30 mg/kg bw; Fig 4B) after DE-83R treatment. Increase in DCX levels was also induced by the same experimental doses (Figure 4C). Prenatal exposure to MeHg at doses of the same magnitude of decaBDE induced similar effects.

DecaBDE or MeHg exposure induces neurinflammatory alterations in stimulated microglia cultures and in prenatally exposed mouse brain.
We also investigated the effects of DE-83R and MeHg on cultured microglia. A significant increase in the circularity index of microglia was observed after exposure to the highest dose of DE-83R (Fig 5B). MeHg induced significant decrease in microglial cell area (Fig 5A) and increase in circularity (Fig 5B) down by 1.33 nM. Cotreatments by combination of the compounds at the highest tested concentrations induced striking activation of microglia, as shown by the large reduction of cell area and increased circularity ($p<0.001$ for both) compared to control cells (Fig 5A,B).
Quantification of CD68 (marker of microgliosis) and GFAP was performed in fetal brains to detect activated microglia or astrocytes, respectively. DE-83R induced increase in CD68 levels by 30 mg/kg bw and in GFAP levels by 0.3 mg/kg (Fig 6A, B). Significant increase in CD68 and GFAP levels was induced also by MeHg. Specific activation of microglia or astrocytes was also evaluated in different brain regions (i.e., hippocampus and cerebellum) by immunohistochemistry for Iba-1 or GFAP on paraffinized brain slices. DE-83R (30 mg/kg bw) induced significant increase in Iba-1- or GFAP-positive cell density in the hippocampus (Fig 7A and B, respectively). Furthermore, DE-83R exposure selectively induced increase in Iba-1-positive cell density (but not GFAP) in the cerebellum (Fig 7C, D).
Taken together, our results suggest that prenatal exposure to low, sublethal concentrations of decaBDE, similar to those reported in human fetal tissues, may induce significant alterations in mammal neurodevelopment. This could be of relevance for the fetotoxic risk assessment for this group of contaminants, especially in light of recent epidemiological studies that clearly correlate the reported increase in PBDEs concentrations in maternal fluid with neurobehavioral disorders.

References

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5. De Paola, et al. Mol Med, 2012 18:971–981.
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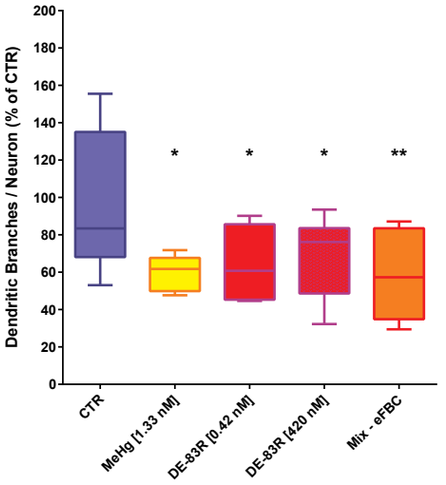
Figure 1. DE-83R and MeHg reduce neuron arborization

A



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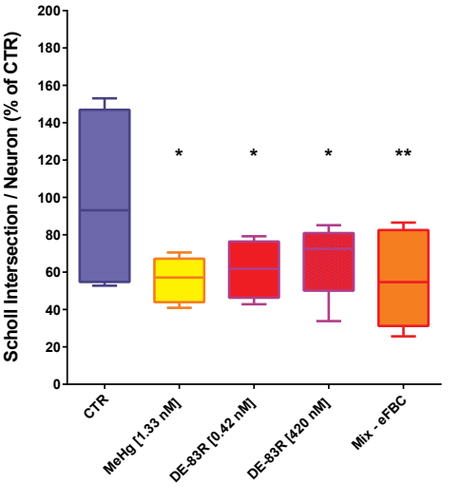

Figure 2. DE-83R increase doublecortin expression

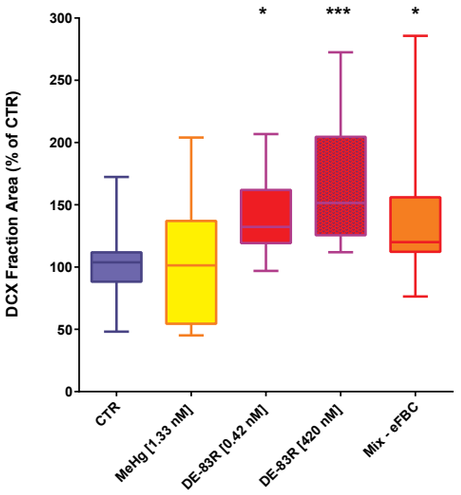

Figure 3. DE-83R and MeHg decrease synaptophysin expression

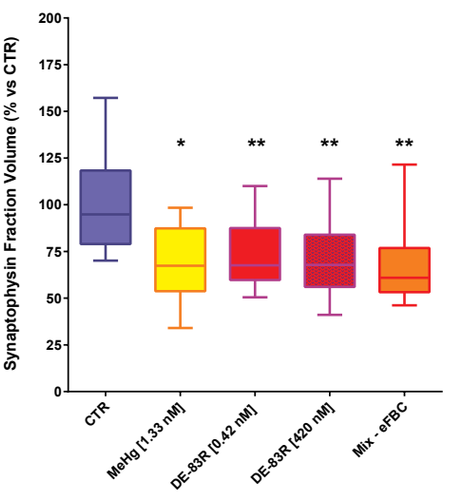

Figure 4. Prenatal exposure to DE-83R or MeHg impairs the levels of neurodevelopmental markers

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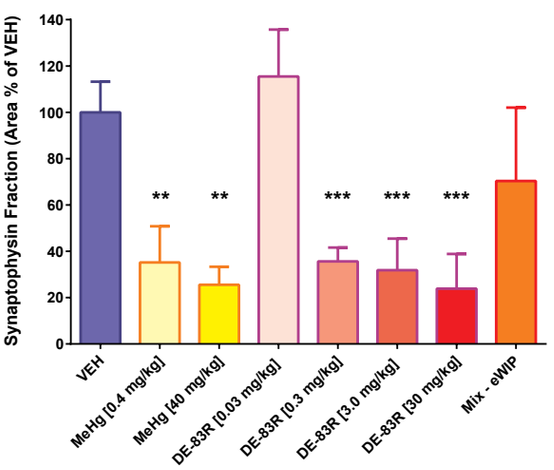

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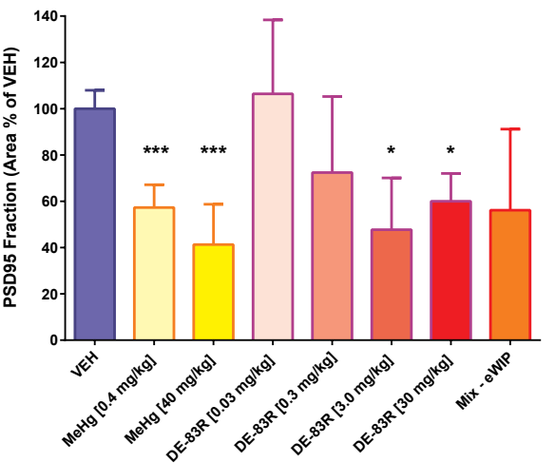

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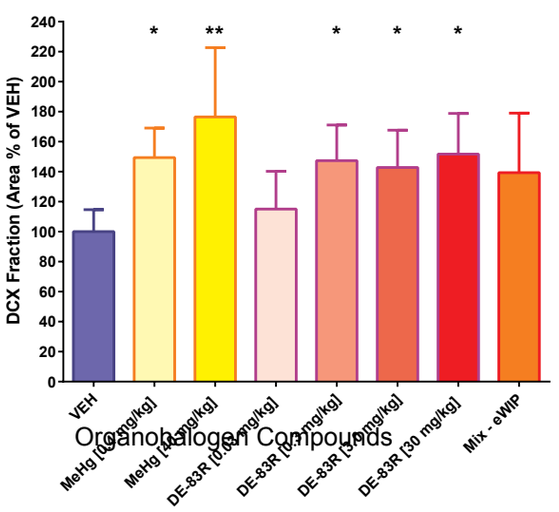
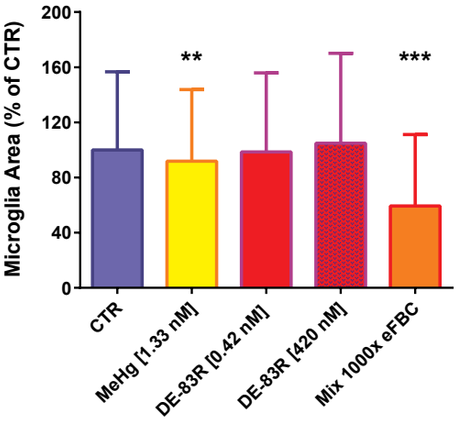


Figure 5. DE-83R and MeHg induce pro-inflammatory morphological changes in cultured microglia

A



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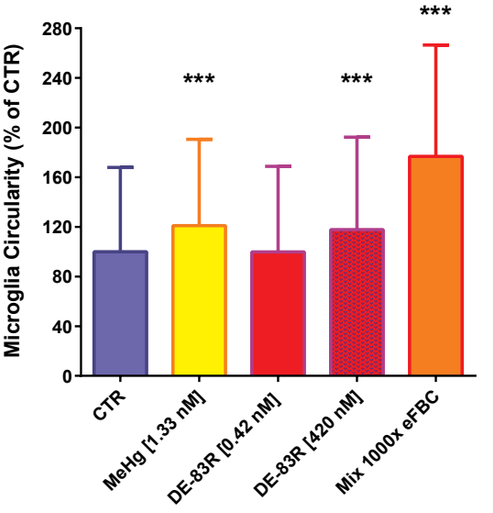

Figure 6. Prenatal exposure to DE-83R or MeHg induces increase in astrocitosis and microgliosis marker levels

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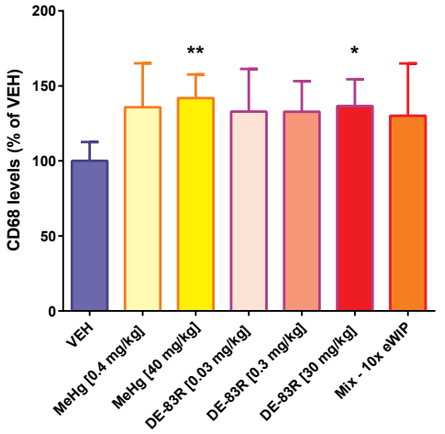

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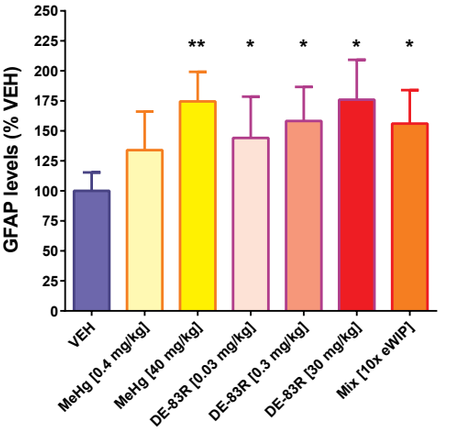

Figure 7. Prenatal exposure to DE-83R or MeHg induces increase in the density of microglia in hippocampus and cerebellum

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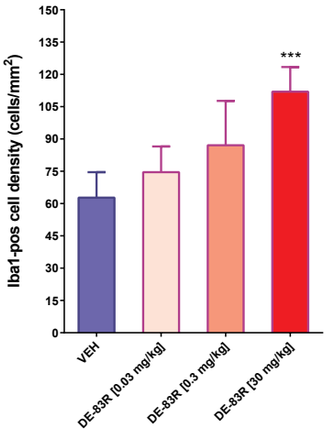

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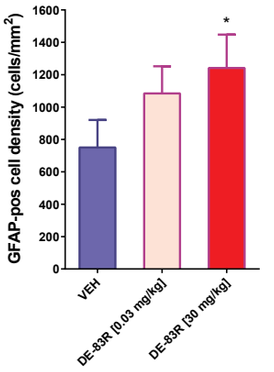

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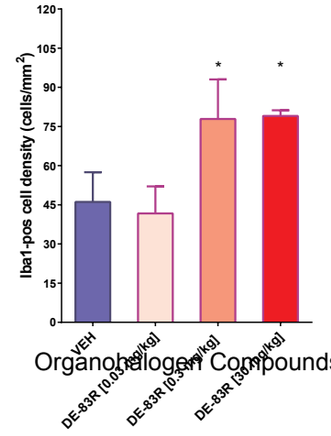

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