Non–Dioxin-like Polychlorinated Biphenyls Interfere with Neuronal Differentiation of Embryonic Neural Stem Cells

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Developmental exposure to food contaminants, such as polychlorinated biphenyls (PCBs), has been considered as a possible cause of neurodevelopmental disorders. We have investigated the effects of noncytotoxic concentrations of PCBs 153 and 180 on spontaneous differentiation of rat embryonic neural stem cells (NSCs). Upon removal of basic fibroblast growth factor to induce spontaneous differentiation, cells were exposed to 100nM of the selected PCBs for 48 h and analyzed after 5 days. Both PCBs 153 and 180 induced a significant increase in the number of neurite-bearing Tuj1-positive cells with a concomitant decrease in proliferating cells, as detected by FUCCI transfection and EdU staining. Measurements of spontaneous Ca\(^{2+}\) oscillations showed a decreased number of cells with Ca\(^{2+}\) activity after PCB exposure, further confirming the increase in neuronal cells. Conversely, exposure to methylmercury (MeHg), which we evaluated in parallel, led to an increased number of cells with Ca\(^{2+}\) activity, in agreement with the previously observed inhibition of neuronal differentiation. Analysis with quantitative PCR of the Notch pathway revealed that PCBs have a repressive action on Notch signaling, whereas MeHg activates it. Altogether, the data indicate that nanomolar concentrations of the selected non–dioxin-like PCBs and MeHg interfere in opposite directions with neuronal spontaneous differentiation of NSCs through Notch signaling. Combined exposures to PCBs and MeHg resulted in an induction of apoptosis and an antagonistic interaction on spontaneous neuronal differentiation. NSCs are further proven to be a valuable model to identify potential developmental neurotoxicants.

Key Words: PCBs; MeHg; neurodevelopmental toxicity; differentiation; Notch signaling; coexposure.

There is an increasing concern about the apparent raising in the frequency of neurodevelopmental disorders, such as attention deficit hyperactive disorder, learning disabilities, and autism. Besides genetic and socioeconomic aspects, environmental factors, including developmental exposures to industrial chemicals entering the food chain, are discussed as possible causes for neurodevelopmental disabilities (Grandjean and Landrigan, 2006).

Among the contaminants abundantly found in food sources, there are the polychlorinated biphenyls (PCBs). The major emission of PCBs in the environment has been related to the use of PCB mixtures in electrical equipment, but coal combustion, steel melting, and waste incineration are also among the possible sources (Safe, 1994). Although PCBs were banned between the 1970’s and 1980’s in most industrialized countries because of their alleged carcinogenicity (World Health Organization, 2003), they are still present in the environment. Depending on the structural characteristics, such as the specific pattern of chlorine substitution in para and meta or ortho positions, PCBs are classified as non–ortho-substituted dioxin-like (DL) or ortho-substituted non–dioxin-like (NDL) congeners (Stenberg and Andersson, 2008). DL-PCBs have high affinity for the aryl hydrocarbon receptor and are regarded to be highly toxic. NDL-PCB congeners are generally considered less toxic, but the nervous system has appeared to be one of their most sensitive targets (Faroon et al., 2001). Epidemiological studies have shown that perinatal exposure to PCBs is associated with an increased amount of abnormally weak reflexes, reduced learning and memory ability, lower IQ scores, and attention deficits (Ribas-Fito et al., 2001; Seegal, 1996; Weisglas-Kuperus, 1998). In addition, PCBs act as endocrine disruptors by interacting with endocrine targets, such as the thyroid, steroid, and retinoid hormone systems (see Winnike et al., 2002). The developmental neurotoxic potential of PCBs has been confirmed by several experimental studies showing behavioral alterations mainly in the motor and cognitive domains (Boix et al., 2011; Bushnell et al., 2002; Piedrafita et al., 2008b; Roege et al., 2000; Seegal, 1996; Vitalone et al., 2008). At the cellular level, PCB exposure has been associated to disruption of Ca\(^{2+}\) homeostasis (Fonnun et al., 2006; Inglefield and Shafer, 2000; Kodavanti and Tilson, 2000) and induction of oxidative stress in neurons (Costa et al., 2007; Dreiem et al., 2009; Lee and Opanashuk, 2004).
Moreover, NDL-PCBs have been suggested to have membrane-disrupting properties (Tan et al., 2004). Still, the cellular mechanisms behind PCBs developmental neurotoxicity remain unclear, and because many of the available data derive from studies based on PCB mixtures, the information on the effects of single PCBs is still very limited.

During the last 10 years, we have implemented the use of rodent and human neural stem cell (NSCs) cultures as in vitro models for developmental neurotoxicity testing. Using rat embryonic NSCs, we have been able to show that levels of methymercury (MeHg), lower than those found in the umbilical cord blood of pregnant women in the general Swedish population (nanomolar range), inhibits spontaneous neuronal differentiation (Tamm et al., 2006, 2008). The purpose of the present study was to investigate the effects of low concentrations of pure NDL-PCBs on the process of NSC neuronal differentiation as compared with MeHg. We focused on two specific congeners, PCBs 153 and 180, the dominating NDL-PCBs because they are among the most abundant in food and in human milk and blood (EFSA, 2010; Fängström et al., 2005; Fantore et al., 2008; Inoue et al., 2006; Llop et al., 2010). Because food can be contaminated by PCBs and MeHg concomitantly, we investigated the effects of combined exposures.

MATERIALS AND METHODS

Chemicals. PCBs 153 and 180 were purchased from Neosync Inc., Possible impurities, e.g., polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/Fs) and DL-PCBs, were removed by applying the PCBs dissolved in n-hexane on an active carbon column and collecting them after elution with n-hexane as described by Danielsson et al. (2008). The purified PCBs (impurity levels less than picograms per gram) were dissolved in purity-checked dimethyl sulfoxide (DMSO). MeHg (methylmercury (II) hydroxide) was purchased from Alfa Chemicals (ALFA, Johnson Matthey, Karlsruhe, Germany). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). Poly-L-ornithine, fibronectin, and the thymidine analog 5-ethyl-2′-deoxyuridine (EdU) were purchased from Sigma (Stockholm, Sweden). The γ-secretase inhibitor DAPT was obtained from Calbiochem. Basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). All other chemicals for cell culture were supplied by Life Technologies (Gibco BRL, Grand Island, NY).

Cell culture procedures and experimental treatments. Primary cultures of NSCs were obtained from embryonic cortices dissected in Hanks’ Balanced Salt Solution (HBSS) from timed-pregnant Sprague-Dawley rats (Harlan Laboratories, The Netherlands) at embryonic day 15 (E15) (the day of copulatory plug was defined as E0). The tissue was gently mechanically dispersed, and meninges and larger cell clumps were allowed to sediment for 10 min. The cells were plated at a density of 40,000/cm² on dishes precoated with poly-L-ornithine and fibronectin. Cells were maintained in enriched N2 medium with ornithine and fibronectin, and grown in the presence of bFGF. Two days after the passing, the medium was changed with no addition of bFGF to promote spontaneous differentiation. Upon withdrawal of bFGF, cells were exposed once to 25–100nM PCB 153, 25–100nM PCB 180, or 5nM MeHg for 48 h. For combined exposure experiments, the cells were exposed to 5nM MeHg and 100nM PCB 153 or 180 for 48 h. Afterward, the medium was changed every second day and the cells were allowed to differentiate for 7 days. PCB 153 (stock concentration 100μM) and PCB 180 (stock concentration 100μM) were diluted in DMSO and control cells were exposed to the same amount of DMSO (0.1%). MeHg (stock concentration 5μM) was diluted in water. All experiments and measurements were performed in triplicate and repeated at least three times unless stated otherwise.

Trypan blue assessment of cell viability and total cell number. Cells were harvested by scraping as described previously; then, an aliquot of the cell suspension was mixed with an equal volume of 0.4% trypan blue in PBS. Cells were scored at the phase contrast microscope using a Neubauer-improved counting chamber. Cells with damaged cell membrane stain blue (dead cells), whereas cells with plasma membrane integrity, which prevents the dye entry, remain unstained (healthy cells and apoptotic cells). For the quantification of total cell number, at least 1,000,000 cells (unstained cells plus if present blue cells) were counted.

Detection of apoptotic cells. To evaluate nuclear morphology, NSCs were grown on poly-L-ornithine/fibronectin-coated coverslips and fixed in 4% paraformaldehyde (PF) (Sigma-Aldrich, Sweden) for 1 h at 4°C. After washing with PBS, cells were stained with Hoechst 33342 (1 μg/ml) for 5 min at room temperature (RT) and then rinsed with PBS. After mounting, cells were analyzed with a fluorescent microscope scoring at least 100 nuclei in five different fields for each coverslip. Apoptotic cells were identified by the condensed chromatin.

Immunocytochemistry. NSCs were fixed in 4% PF for 1 h at 4°C followed by washing in PBS. Primary antibodies were diluted in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (Boehringer Mannheim, Bromma, Sweden). NSCs fixed on coverslips were incubated with one of the following primary antibodies; mouse anti-nestin (1:200; Chemicon); rabbit anti-glia fibrillary acidic protein (GFAP, 1:800; Dakocytomation), and mouse anti-tubulin III (TuJ1, 1:400; Convance), overnight in a humid chamber at 4°C. Cells were then rinsed with PBS and incubated with appropriate secondary FITC- or Texas-Red-conjugated antibodies for 1 h at RT (1:200; Alexa, Molecular Probes, Invitrogen, Carlsbad, CA). Cell nuclei were counterstained with Hoechst 33342 (1 μg/ml). After rinsing with PBS, coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Inc.). Images were captured using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu digital camera (C4742-95-10sc; Hamamatsu Photonics Norden AB, Solna, Sweden). For quantification, at least 100 nuclei in five different fields for each coverslip were scored and the percentage of cells positive for the different markers was calculated.

Cell cycle analysis. Cells were transfected using the fluorescent ubiquitination cell cycle indicator (FUCCI) plasmids (kind gift from Dr. Atsushi Miyawaki) to analyze cell cycle progression following treatments (Sakane-Sawano et al., 2008). The two plasmids encoding green and red fluorescent proteins fused with E3 ligase substrates indicate whether live cells are in G1 (non-proliferating) or S/G2/M (proliferating) phase. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol and the efficiency was > 10%. The number of proliferating (green) and non-proliferating (red) nuclei was counted 48 h after transfection.

5-Ethynyl-2′-deoxyuridine (EdU) incorporation, EdU is a thymidine analog, containing a terminal alkyne group that readily gets incorporated into cellular DNA during the S-phase. The terminal alkyne group is then detected through its reaction with fluorescent azides. Cells were spontaneously differentiated and exposed to the toxics as previously described, and 1 h prior to fixation, 10μM EdU was added to the culture media. After rinsing with PBS, cells were stained by incubation with a freshly prepared mix of 100nM Tris (pH 8.5), 1mM CuSO4, 10μM Alexa 488-azide (Invitrogen), and 100nM ascorbic acid for 30 min. After staining, the cells were washed with PBS, counterstained with Hoechst 33342, and mounted. At least 100 nuclei were...
ANOVA. *Significantly different from the respective control. Scale bars: B–E (I). Values are shown as mean ± 100nM PCB 153 (C and G), 100nM PCB 180 (D and H), or 5nM MeHg (E and F) with Tuj1 (B–E) or nestin (F–I). Cells were exposed to DMSO (B and F), PCB 153 (C and G), PCB 180 (D and H), or MeHg (E and F) for 7 days. After fixation, cells were fixed and stained with the early neuronal marker Tuj1 or the NSC marker nestin antibodies and analyzed by fluorescence microscopy. Semiquantitative analysis showed that in comparison with DMSO-exposed groups. For comparisons of two groups, Student’s t-test was applied. p Values < 0.05 were considered statistically significant.

Immunoblotting. NSCs were harvested by scraping, centrifuged, and washed with PBS. Then, cells were sonicated in a solution containing 1mM Pefabloc (Boehringer Mannheim), 10mM EDTA, and 2mM DTT in PBS. The protein content was determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). After adding sample buffer (0.4% SDS, 4% glycerol, 1% β-mercaptoethanol, and 12.5mM Tris-HCl, pH 6.8), 100 µg total protein was boiled for 5 min and subjected to 6% SDS-polyacrylamide gel electrophoresis followed by electroblotting to nitrocellulose membrane. Membranes were incubated overnight at 4°C with polyclonal primary antibody against total Notch1 (C-20-R, 1:1000; Santa Cruz). Equal protein loading was verified with rabbit anti-GAPDH (1:3000; Nordic Biosite, Taby, Sweden). The membranes were rinsed and incubated with horseradish peroxidase–conjugated secondary antibodies (1: 10,000; Pierce, Rockford, IL) for 1 h at RT. Then, membranes were rinsed again, developed with ECL reagents (Amersham), and exposed to x-ray autoradiography films (Fuji).

Ca²⁺ measurements. Cells were incubated (30 min at 37°C in 5% CO₂) in cell culture medium containing 5µM Fluo-3/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). After rinsing the cells, KREBS-Ringer’s solution was added (119mM NaCl, 2.5mM KCl, 1mM NaH₂PO₄, 2.5mM CaCl₂, 1.3mM MgCl₂, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 11mM D-glucose). Petri dishes were transferred to a Zeiss LSM 510 META scanning laser confocal microscope equipped with a ×20/1.0 dipping lens (Zeiss), and images were acquired at 0.2 Hz. To determine the number of active cells, a spontaneously active cell was defined as a cell that displayed at least two well-defined spontaneous Ca²⁺ peaks in 10 min, where each peak value was an increase in Ca²⁺ of more than 15% compared with the baseline. At the end of the experiment, 100µM glutamate was bath applied. Spectral analysis of Ca²⁺ oscillations and response to glutamate was performed with MATLAB software as described previously (Uhlén, 2004). All measurements were performed in three different preparations per each experimental condition.

Extraction of total RNA, complementary DNA synthesis, and quantitative real-time PCR (qPCR) Total RNA was isolated from NSCs using the RNasey Mini Kit (Qiagen, VWR, Stockholm, Sweden). Integrity and concentration of extracted RNA was measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was prepared using 1 µg total RNA and 0.5 µg of Oligo-dT primer following the instructions of Superscript II First-Strand cDNA Synthesis Kit (Invitrogen Inc.). Amplification reactions were performed with 1 µl cDNA, SYBR Green Mix (Applied Biosystems), and 0.2µM of forward and reversed primers. The following PCR primers were used: Hes5 RP atgtctcaagccagagaa LP tagctcggtgtcagccttt, Math1 RP ttccagacaaaggtgagat LP tgacagctttcttggtt, and CXXC1 RP gaggtctgtgggtgtccact LP atctgtggatgggttgtgctt. The reaction volume was adjusted to 25 µl with DEPC water. Negative control reactions contained water instead of cDNA as template. Quantitative real-time PCR was performed using an Applied Biosystems Fast Real-Time PCR System with SDS version 2.1 software (Applied Biosystems). The PCR cycle conditions were 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min (40 cycles). To evaluate the amplification of a specific sample, final melting curve (from 60°C up to 95°C) was added under continuous fluorescence intensity measurement. For quantification of the relative messenger RNA (mRNA) levels, the following equation provided by the Perkin-Elmer Instruction Manual of 1997 was utilized:

\[ \text{DD}_{\text{relative change}} = \text{CT} \quad \text{of the target mRNA} - \text{CT} \quad \text{of the control mRNA} \]

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where CT is the point (cycle number) at which the amplification plot crosses the threshold and ΔCT = (CT of the target mRNA – CT of the control mRNA), where the amount of target/control gene has been normalized to CXXC1.

Statistical analysis. All experiments were performed in triplicates and repeated at least three times. One-way ANOVA followed by Fisher’s protected least significant difference was used for comparisons between control and exposed groups. For comparisons of two groups, Student’s t-test was applied. p Values < 0.05 were considered statistically significant.
RESULTS

PCBs 153 and 180 Enhance Spontaneous Neuronal Differentiation

To investigate the putative effects of very low concentrations of NDL-PCBs on the process of spontaneous differentiation of embryonic NSCs, we exposed the cells upon removal of bFGF to 25–100nM PCBs 153 or 180 for 48 h and performed the analyses after 5 days. In parallel experiments, we exposed NSCs to 5nM MeHg. None of the tested concentrations of PCBs or MeHg induced necrotic or apoptotic cell death (data not shown). We observed that exposure to 100nM PCB 153 or 180, but not lower concentrations, resulted in a significant increase in the number of neurite-bearing TuJ1-positive cells (Figs. 1A, 1C, and 1D) as compared with control cells (Figs. 1A and 1B), whereas, as expected, MeHg decreased significantly the number of TuJ1-positive cells (Figs. 1A and 1E). Concomitantly, PCBs decreased significantly the number of Nestin-positive cells (PCB 153, 67.8 ± 7.2; PCB 180, 69.7 ± 10.7) (Figs. 1G and 1H) compared with control (Fig. 1F) (DMSO 79.5 ± 11.2), whereas MeHg increased it (Fig. 1I) (control 78.5 ± 9.7; MeHg 92.2 ± 5.0). No significant changes in the number of GFAP-positive cells were observed in any of the treated groups as compared with their controls (DMSO, 7.2 ± 1.6; PCB 153, 7.4 ± 1.4; PCB 180, 7.5 ± 1.7; control 7.1 ± 1.4, and MeHg 6.6 ± 1.2).

Exposure to PCBs 153 or 180 Results in Decreased NSCs Proliferation

To check whether the increased differentiation was associated with a decrease in cell proliferation, NSCs were transfected with two plasmids encoding fluorescent proteins (FUCCI). The percentage of non-proliferating and proliferating cells was calculated after 7 days of differentiation by counting the number of nuclei in G1 phase or in S/G2/M-phase (80–160 cells, 15–25 fields of view, 3 experiments). The number of proliferating cells was significantly decreased in PCB-exposed cells (100nM), whereas it was increased in MeHg-exposed cells (5nM) (Fig. 2A; Supplementary figure. 1). Also, EdU incorporation experiments showed that a significantly lower number of nuclei were stained with EdU in PCB-exposed NSCs opposite to MeHg-exposed cells that had more positive nuclei (Fig. 2B). In agreement with these results, PCBs significantly decreased the total cell number, whereas MeHg increased it as detected by trypan blue staining (Fig. 2C).

PCBs Decrease the Number of Cells with Ca^{2+} Activity

After 7 days of differentiation, cells were loaded with Fluo3-AM and Ca^{2+} imaging was performed with a confocal microscope. Exposure to 100nM PCB 153 or 180 decreased the number of cells showing spontaneous Ca^{2+} activity, whereas 5nM MeHg caused an increase (Fig. 3). Differentiated neurons respond to glutamate; therefore, we examined the response of NSCs to glutamate by directly adding it in the culture medium of the control and exposed cells at day 7. Both PCBs increased the number of glutamate-responsive cells, but statistical significance was reached only in the PCB 153–exposed NSCs (DMSO, 79.2 ± 13.0; PCB 153, 91.6 ± 4.7; and PCB 180, 91.1 ± 10.4). MeHg decreased significantly the number of glutamate-responsive cells (control 93.9 ± 3.8, MeHg 71.4 ± 18.0) in agreement with the observed decrease of neuronal differentiated cells (Supplementary figure. 2).

Notch Signaling Is Repressed by Exposure to PCBs

Signaling through Notch plays a key role in NSCs proliferation and differentiation. We therefore analyzed the expression of total Notch1 by immunoblotting. NSCs exposed to 100nM PCBs showed higher levels of Notch1, whereas MeHg-exposed cells (5nM) showed lower levels (Figs. 4A and 4B). To confirm the involvement of Notch in the observed effects on NSCs, we checked the activation of downstream Notch target genes by measuring the mRNA levels of Hes5 and Math1, antineuronal and pro-neuronal transcription factors, respectively, at different days (days 1–7) after the exposures. We observed a significant downregulation of Hes5 and an upregulation of Math1 in PCBs 153– and 180-exposed cells after 3 days of differentiation (Fig. 4C). Conversely, at the same day, a significant upregulation of Hes5 and a downregulation of Math1 were detected in MeHg-exposed cells (Fig. 4C). To further prove the relevance of the Notch signaling, we blocked the cleavage of Notch receptors by using the γ-secretase inhibitor DAPT (2.5mM) daily administrated to NSCs undergoing spontaneous differentiation. As shown in Figure 4D, only the effects of MeHg could be prevented by inhibiting the Notch pathway supporting the idea that in NSCs both PCBs 153 and 180 exert a repressive action on Notch signaling opposite to MeHg that induces activation.

Effects of Combined Exposure to PCB 153 or 180 and MeHg

To evaluate the effects of combined exposure on spontaneous neuronal differentiation, we exposed NSCs to 100nM of PCB 153 or 180 in combination with 5nM MeHg for 48 h. As mentioned before, at these concentrations, each single toxicant induced neither apoptotic nor necrotic cell death as compared with control NSCs. Conversely, the exposure to both combinations (PCB 153 + MeHg or PCB 180 + MeHg) significantly increased the number of cells undergoing apoptosis (Fig. 5A). The effects of the combined exposures on neuronal differentiation pointed to an antagonistic interaction between PCBs and MeHg (Fig. 5B).

DISCUSSION

We designed this study to investigate the effects of pure NDL-PCB 153 and PCB 180 on spontaneous neuronal differentiation of embryonic NSCs. The experiments were performed on primary cultures of NSCs prepared from cortices of E15 rat embryos, a model that we have implemented for
resting concentration and a high extracellular concentration, thus fluctuations can be generated by a relatively small increase in the cytosol. These fluctuations give rise to signals that convey vital information controlling cellular processes (as reviewed by Uhlen and Fritz, 2010). Ca\(^{2+}\) has a critical role throughout the cell cycle, especially during the early G1 phase and at the G1/S and G2/M transition phases (Pande et al., 1996). Spontaneous Ca\(^{2+}\) oscillations have been implicated in the progression of cell cycle in embryonic carcinoma cells and adult stem cells (Resende et al., 2010) and in the regulation of migration and neuronal differentiation (Gomez et al., 1995; Gu and Spitzer, 1995; Komuro and Rakic, 1996). Although spontaneous Ca\(^{2+}\) signals in undifferentiated cells may persist for many days, they become less frequent as the stem cells differentiate into mature neurons. This has been shown in mouse primary culture of NSCs from embryonic day 14 where both global and local spontaneous Ca\(^{2+}\) signals were shown to be more frequent at early stages of neural precursor differentiation (Ciccoldini et al., 2003). Interestingly, this study also showed that there was a trend for an increasing number of neurons to be glutamate responsive with time in culture, meaning that at later stages of differentiation neurons become more competent at Ca\(^{2+}\) signaling. Similarly, in our experimental model, the number of glutamate-responsive cells in PCB-exposed NSCs was also increased, although for PCB 180 the difference was not statistically significant. As to be expected, MeHg decreased significantly the number of glutamate-responsive cells.

The Notch signaling pathway has been implicated in a wide variety of essential cellular events such as proliferation, migration, differentiation, and neurite outgrowth (Artavanis-Tsakonas et al., 1999). In mammals, the main components of Notch signaling includes the transmembrane Notch receptors (Notch 1–4), the ligands (e.g., Delta-like and Jagged), and Notch-responsive transcription factors (e.g., CBFI/Su(H)/Lag1) (Hurlbut et al., 2007). The mature Notch receptor consists of a large extracellular domain and a smaller transmembranal domain (Rand et al., 2000). Upon ligand binding, Notch is cleaved at the extracellular domain by the ADAM family of metalloproteases, whereas the intracellular part is cleaved by the action of gamma-secretases (Bland et al., 2003; Kageyama et al., 2005; Rand et al., 2000).

The Notch intracellular domain (NICTD) is then released and subsequently translocated to the nucleus. In the nucleus, NICTD can bind directly to CSL, a transcriptional corepressor that in the absence of Notch activation recruits histone deacetylase and corepressor components such as N-CoR in a cell context-specific manner, but upon NICTD binding, N-CoR is displaced and CSL is converted into a transcriptional activator resulting in activation of downstream Notch target genes (Hurlbut et al., 2007; Kageyama et al., 2009). Hes proteins, one target of CSL, are transcription factors that exert repressive effects on transcriptional activity and neuronal differentiation, whereas Math1 are positive regulators of neuronal differentiation. Whether differentiation may proceed or not depends on a balance between positive and negative regulators (Kageyama et al., 2005). In
The present study, we found downregulation of Hes5 and upregulation of Math1 at the mRNA level in parallel with upregulation of total Notch1 at the protein level in both PCB 153– and 180-exposed cells. In contrast to our data, the commercial PCB mixture Aroclor 1254 has been shown to increase the expression of Hes1 and Hes5 in the fetal rat brain.

**FIG. 3.** To measure spontaneous Ca$^{2+}$ oscillations, control and exposed NSCs at day 7 were loaded with Fluo3-AM, and Ca$^{2+}$ imaging was performed with a confocal microscope. Graphs showing 10 single cell Ca$^{2+}$ recordings of DMSO (A), 100nM PCB 153 (B), 100nM PCB 180 (C), Cont (D), and 5nM MeHg-exposed cells (E). (F) Exposure to PCBs (PCB 153 and PCB 180, 100nM) decreased the number of cells showing spontaneous Ca$^{2+}$ activity, whereas MeHg (5nM) increased it. Values are shown as mean ± SEM. Statistical analysis was performed by Student’s t-test. *Significantly different from control.
The presence of different PCBs in the exposure mixture as well as the modality and timing of the administration (from E6 to E16 when the embryos were taken) might explain the discrepancy.

In contrast to PCBs and in agreement with our previous results (Tamm et al., 2008), we found activation of the Notch signaling by MeHg, as shown by the significant upregulation of Hes5, downregulation of Math1, decreased expression of total Notch1, and the blocking effect of $\gamma$-secretase inhibitor DAPT. Interestingly, a direct link between Notch and G1/S cell cycle phase has been identified in embryonic stem cells resulting in Notch1-induced Cyclin D1–dependent increased proliferation (Das et al., 2010). These findings are in line with our results, which show that low concentrations of MeHg activates the Notch pathway, inhibits neuronal differentiation, and stimulates proliferation, opposite to PCBs 153 and 180.

The results from the combined exposure experiments are intriguing. Although a synergistic interaction occurred with regard to the induction of apoptotic cell death, we observed an antagonistic interaction on spontaneous neuronal differentiation of NSCs. Experimental in vitro and in vivo studies on combined exposure to PCBs and MeHg have shown antagonistic, synergistic, or additive effects depending on the experimental model, parameters considered and modality of exposure (Bemis and Seegal, 2000; Costa et al., 2007; Johansson et al., 2006; Piedrafita et al., 2008a; Tofighi et al., 2011). Interestingly, in a cohort study from Faroe Island, no PCB effects were apparent in children with low-mercury exposure, whereas children with high mercury showed PCB-associated neurobehavioral deficits, indicating a possible interaction between the two neurotoxic agents (Grandjean et al., 2001). Considering the relevance of coexposures to environmental contaminants, more studies are necessary to clarify the mechanisms involved in these complex chemical/biological multiple interactions.

In conclusion, noncytotoxic nanomolar concentrations of both NDL-PCBs and MeHg interfere with the spontaneous neuronal differentiation of NSCs and, when combined, with NSC survival. The adverse impact that these alterations may have on the development of the nervous system could have long-lasting consequences and play a critical role in the onset of the functional and behavioral changes observed in humans and experimental animals. Environmental contaminants, such as PCBs and MeHg, may affect the development of the nervous system through the Notch signaling pathway, and their combined exposure may have synergistic or antagonistic effects on neuronal differentiation. Further studies are needed to understand the mechanisms involved in these complex interactions.
as PCBs, are considered among possible causes of neurodevelopmental disorders, and more information is needed to understand the mechanisms behind the neurotoxic effects especially during development. NSCs appear to be a relevant model for in vitro neurotoxicity studies, and parameters such as cell proliferation and differentiation are sensitive endpoints to identify substances with developmental neurotoxic potential and to identify relevant mechanistic processes that may lead to novel preventive and protective strategies.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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