Polychlorinated bisphenyls (PCBs) are ubiquitous environmental chemicals that accumulate in adipose tissues over the food chain. Epidemiologic studies have indicated that PCBs influence brain development. Children who are exposed to PCBs during development suffer from neuropsychologic deficits such as lower full-scale IQ (intelligence quotient), reduced visual recognition memory, and attention and motor deficits. The mechanisms leading to these effects are not fully understood. It has been speculated that PCBs may affect brain development by interfering with thyroid hormone (TH) signaling. Because most of the data are from animal studies, we established a model using primary normal human neural progenitor (NHN) cells to determine if PCBs interfere with TH-dependent neural differentiation. NHNP cells differentiate into neurons, astrocytes, and oligodendrocytes in culture, and they express a variety of drug metabolism enzymes and nuclear receptors. Like triiodothyronine (T3), treatment with the mono-ortho-substituted PCB-118 (3,3',4,4',5-pentachlorobiphenyl; 0.01–1 μM) leads to a dose-dependent increase of oligodendrocyte formation. This effect was congener specific, because the coplanar PCB-126 (3,3',4,4'-pentachlorobiphenyl) had no effect. Similar to the T3 response, the PCB-mediated effect on oligodendrocyte formation was blocked by retinoic acid and the thyroid hormone receptor antagonist NH-3. These results suggest that PCB-118 mimics T3 action via the TH pathway.  

Key words: NH-3, NHNP cells, oligodendrocyte, PCB, retinoic acid, thyroid hormone receptors.

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Research

Polychlorinated Biphenyls Disturb Differentiation of Normal Human Neural Progenitor Cells: Clue for Involvement of Thyroid Hormone Receptors

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Polychlorinated biphenyls (PCBs) are anthropogenic industrial chemicals, the production of which was banned in the 1970s because of their presumed carcinogenicity (Chana et al. 2002). However, these chemicals are still present in the food chain; they accumulate in animal and human tissues and are among the most abundant persistent organic pollutants found in humans (DeKoning and Karmaus 2000; Kim et al. 2004). Depending on their degree of chlorination, they are metabolized to adipose tissues over the food chain. Epidemiologic studies have indicated that PCBs influence brain development. Children who are exposed to PCBs during development suffer from neuropsychologic deficits such as lower full-scale IQ (intelligence quotient), reduced visual recognition memory, and attention and motor deficits. The mechanisms leading to these effects are not fully understood. It has been speculated that PCBs may affect brain development by interfering with thyroid hormone (TH) signaling. Because most of the data are from animal studies, we established a model using primary normal human neural progenitor (NHN) cells to determine if PCBs interfere with TH-dependent neural differentiation. NHNP cells differentiate into neurons, astrocytes, and oligodendrocytes in culture, and they express a variety of drug metabolism enzymes and nuclear receptors. Like triiodothyronine (T3), treatment with the mono-ortho-substituted PCB-118 (3,3',4,4',5-pentachlorobiphenyl; 0.01–1 μM) leads to a dose-dependent increase of oligodendrocyte formation. This effect was congener specific, because the coplanar PCB-126 (3,3',4,4'-pentachlorobiphenyl) had no effect. Similar to the T3 response, the PCB-mediated effect on oligodendrocyte formation was blocked by retinoic acid and the thyroid hormone receptor antagonist NH-3. These results suggest that PCB-118 mimics T3 action via the TH pathway.  

Key words: NH-3, NHNP cells, oligodendrocyte, PCB, retinoic acid, thyroid hormone receptors.
ethanol at a concentration of 300 mM. Ortho-substituted PCB-118 (2,3',4,4',5-pentachlorobiphenyl), coplanar PCB-126 (3,3',4,4',5-pentachlorobiphenyl (both from Ökometric GmbH, Bayreuth, Germany), all-trans-retinoic acid (RA; Sigma-Aldrich) and the TH antagonist NH-3 (Nguyen et al. 2002) were diluted in DMSO (Sigma-Aldrich) at stock concentrations of 1.53, 1.59, 10, and 10 mM, respectively. Benzo(a)pyrene (BAP; Sigma-Aldrich) was diluted in tetrahydrofuran (10 mM).

**Cell culture and treatment.** NHNP cells were purchased from Cambrex BioScience (Verviers, Belgium) and cultured as neurospheres in NPMM (Neural Progenitor Maintenance Medium; Cambrex BioScience) at 37°C with 5% CO2. Medium was changed every 2–3 days. Upon significant growth (0.7-mm diameter), spheres were chopped with a McIlwaine tissue chopper as previously described (Svendsen et al. 1998); the resultant cubes formed new spheres within hours and were named according to increasing passages after each chopping event (passes 1–7).

For treatment of neurospheres, chemicals were diluted in NPMM to the following final concentrations: 30 nM T3; 0.01 µM, 0.1 µM and 1 µM PCB-118 and PCB-126; 10 µM BAP; 1 µM each RA and NH-3; and 0.065% DMSO. We treated 3–10 spheres with a diameter of approximately 0.4 mm each for 7 days before plating for differentiation. Spheres were treated with each chemical alone or with a cotreatment containing PCB-118 and either NH-3 or RA for 1 week. Differentiation of NHNP cells was initiated by growth factor withdrawal and plating onto poly-D-lysine coated chamber slides (BD Biosciences, Erembodegem, Belgium). Neurospheres were plated in a defined medium consisting of Dulbecco modified Eagle medium (DMEM)/F12 (3:1) supplemented with N2 (Invitrogen GmbH, Karlsruhe, Germany). After differentiating for 2 days, cells were fixed in 4% paraformaldehyde for 30 min and stored in phosphate-buffered saline (PBS) at 4°C until immunostaining was performed.

**Immunocytochemistry.** Fixed slides were washed two times for 5 min each in PBS. Slides were incubated with the following primary antibodies: a) double staining beta(III)tubulin 1:100 and glial fibrillary acidic protein (GFAP) 1:1000 (both from Sigma-Aldrich) in PBS containing 0.3% Triton X-100, or b) mouse antioliog dendrocyte marker O4 1:15 (Chemicon, Temecula, CA, USA) in PBS with 10% goat serum for 1 hr at 37°C followed by three 10-min washes with PBS. We used fluorescein isothiocyanate (FITC)- and/or Rhodamine Red-coupled secondary antibodies (1:100 each; Jackson ImmunoResearch, Dianova GmbH, Hamburg, Germany) for detection by incubating slides for 30 min at 37°C, followed by three 10-min washes with PBS.

### Table 1. Sequences of oligonucleotides used to perform RT-PCRs with NHNP cells as shown in Figure 1.

| Gene | Sequences | Size (bp) | Annealing temperature (°C) | Reference |
|------|-----------|----------|-----------------|-----------|
| β-Actin | FW CCCCAGGCACCAACGCGGCTGATGAT | 263 | 60 | Ihm et al. 2002 |
| NSE  | FW CCCCAGGCACCAACGCGGCTGATGAT | 254 | 60 | Ko et al. 2000 |
| GFAP | FW CCCCAGGCACCAACGCGGCTGATGAT | 206 | 60 | Ko et al. 2000 |

**Figure 1.** Neurosphere plated on poly-D-lysine-coated slides showing differentiation and radial outgrowth of cells out of the sphere after 4 days in culture. Phase contrast image. Bar = 200 µm.
of the NHNP cells. Plating of spheres onto poly-D-lysine-coated chamber slides under withdrawal of growth factors resulted in quick radial outgrowth and differentiation of the cells (Figure 1). After immunostaining, the differentiated cells were identified as neurons, astrocytes, and oligodendrocytes (Figure 2). Furthermore, neurons seem to form a neuronal network.

To determine molecular characterization of NHNP cells, we performed RT-PCRs of cell type-specific genes throughout the first three passages. We could identify typical gene products for the three different cell lineages in undifferentiated neurospheres: neuron specific enolase (NSE) for neurons, GFAP for astrocytes (Figure 3), and proteolipid protein with its splicing variant dm20 (data not shown) for oligodendrocytes. Finding these cell-specific markers in undifferentiated cells implies that specific cell fate is determined before plating and differentiation of cells.

To ascertain if NHNP cells are suitable for neurotoxicologic studies, we characterized them for their expression of genes playing a role in xenobiotic metabolism. The results obtained from undifferentiated neurospheres are shown in Figure 3. NHNP cells express thearyl hydrocarbon receptor (AhR) and the AhR repressor (AhRR), which represent central proteins in the regulation of AhR battery genes. Concerning phase 1 enzymes, we could detect gene products for cytochrome P450 (CYP)1A1, CYP1B1, and CYP2D6, whereas CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 were not expressed. With regard to phase 2 enzymes, NHNP cells do express glutathione S-transferase (GST)M1 and GSTT1, but are abundant for UDP-glucuronosyltransferase (UGT)1A6. Hence, NHNP cells have the ability to metabolize xenobiotics.

Our objective was to investigate endocrine disruption of TH homeostasis in NHNP cells; thus, we studied the expression of genes coding for thyroid hormone receptors (TR), retinoid acid (RAR), and retinoid X receptors (RXR), which are crucial molecules in hormone signal transduction. Undifferentiated NHNP cells express TRα1, β1, and β2, as well as RARα and β and RXRα, β, and γ. Therefore they represent a suitable cell model for investigating thyroid hormone disruption.

**Effects of T3 and PCBs on NHNP cells.**
Our initial goal was to investigate the mechanisms leading to disturbance of human brain development in a human in vitro model. Because disruption of thyroid hormone signaling is suspected to be involved in impairment of intellectual development by PCBs (reviewed by Zoeller and Crofton 2000) and because the timing of oligodendrocyte development seems to be dependent on TH (reviewed by König and Moura 2002), we investigated the occurrence of oligodendrocytes during differentiation of NHNP cells. Therefore, undifferentiated neurospheres were treated with 30 nM T3 for 1 week. After 2 additional days of differentiation, we found a significant increase in the

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**Results**

**Cultivation and molecular characterization of NHNP cells.** Neurospheres were successfully kept in suspension culture over several months. When they exceeded 0.7 mm in diameter, they were passaged by chopping into 0.3-mm cubes. This passaging was performed up to seven times during the lifespan of PBS. In the third wash, we added 0.1 μg/mL Hoechst for nuclear staining. After brief drying, slides were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), covered with cover glass, and sealed with nail polish.

Slides were examined using a fluorescent microscope (Olympus, Hamburg, Germany), and photographs were taken with a ColorView XS digital camera (Olympus). We determined the number of O4-positive oligodendrocytes for each individual sphere by manual counting.

**Statistical analysis.** The counts were approximately lognormally distributed. Therefore, we used the geometric mean and the standard deviation of the geometric mean. The t-test was performed after logarithmic transformation of the values, and each treatment was compared to its respective control. The inhibition values were not logarithmically transformed.

**RNA preparation and reverse transcription polymerase chain reaction.** Total RNA was prepared from 10–15 pooled untreated and undifferentiated spheres (passages 0–2) using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described (Döhr et al. 1995). Sequences and annealing temperatures of the PCR primers are listed in Table 1. Fragments were separated on a 3% agarose gel containing ethidium bromide and visualized under ultraviolet light. We used a 100-bp marker (peqlab, Erlangen, Germany) to estimate the appropriate sizes of the PCR fragments.

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**Figure 2.** Immunocytochemical staining of differentiated NHNP cells. (A) |III|Tubulin-positive neurons (green) and GFAP-positive astrocytes (red); nuclei stained with Hoechst. (B) O4-positive oligodendrocyte.
number of oligodendrocytes formed compared to the medium controls (Figure 4). Treating neurospheres with PCB-118 for 1 week also led to an increase in oligodendrocyte formation, whereas PCB-126 had no effect. It is noteworthy that the solvent DMSO shows some intrinsic effect in this system (Figure 4). Thus, PCB-118 seems to have a TH-like effect in NHNP cells.

**Antagonism of T3 effects with RA and NH-3.** To determine whether the TH-like effect of PCB-118 is mediated by TH receptors, we cotreated NHNP cells with 30 nM T3, 1 µM PCB-118, 1 µM RA, and 1 µM NH-3, or in combination. After 1 week, we counted the number of oligodendrocytes in the neurospheres. Both RA and NH-3 treatment counted the number of oligodendrocytes in the NH-3, or in combination. After 1 week, we counted the number of oligodendrocytes in the neurospheres. Both RA and NH-3 treatment inhibited the formation of oligodendrocytes by T3 and PCB-118 while having no intrinsic activity themselves (Figure 5). These results support the conclusion that PCB-118 acts by interfering with the TR complex.

**Discussion**

It is now generally accepted that developmental exposure to drugs or chemicals can have adverse effects on the structure or function of the nervous system. Identification of such substances resulted mainly from epidemiologic data and animal studies. It is important to develop in vitro approaches because, in some cases, severe species differences can exist (Harry et al. 1998; Tilson 1996). In this article, we characterize an in vitro human neural model. To demonstrate the toxicologic usefulness of this model, we have shown the effects of two different PCB congeners on neural development. Although the ability of PCB congeners to induce cytochrome P450 enzymes has been intensively studied in rats (Parkinson et al. 1983), AhR-dependent toxic equivalency factors were revised at an expert meeting organized by the World Health Organization (van den Berg et al. 1998). In this report, van den Berg et al. (1998) described PCB-118 as a compound with weak dioxin-like activity and PCB-126 as a congener with strong dioxin-like properties. The present findings demonstrate that an individual PCB congener known to widely contaminate human populations can alter the course of neural differentiation in primary NHNP cells. This effect was restricted to PCB-118, which has weak dioxin-like activity, and was not observed following treatment with PCB-126, a dioxin-like congener, despite the fact that these cells express the dioxin receptor (AhR). Moreover, the effect of PCB exposure on oligodendrocyte differentiation was similar to the effect of T3 and could be blocked by the T3 antagonist NH-3. Therefore, these findings suggest that nondioxin-like PCB congeners such as PCB-118 may directly interfere with TH signaling in the developing human brain, altering the course of neural differentiation and potentially accounting for the observation that exposure to PCBs is linked to cognitive deficits in the human population.

We are the first to establish a human primary cell model for investigating endocrine disruption in neural development. NHNP cells, which have the ability to differentiate into the three major cell types of the human brain—neurons, astrocytes, and oligodendrocytes (Figure 2)—formed the basis of this model. The number of oligodendrocytes was relatively low, with approximately 30% of the differentiated cells being neurons and approximately 70% appearing as astrocytes (data not shown). Other laboratories have reported a distinct distribution pattern of neurons and glia cells in human neurospheres (Buc-Caron 1995; Caldwell et al. 2001; Kanemura et al. 2002; Messina et al. 2003; Piper et al. 2001). These differences may be due to culture conditions, ages of the embryos/fetuses, or the brain areas from which the cells were prepared. Nevertheless, the low abundance of oligodendrocytes in NPHH cells provides a very sensitive system to identify agents that induce their differentiation.

Two important features of our in vitro model support their use in studies of chemical exposure on neurodevelopment: their xenobiotic metabolic capacity and their TH signal transduction machinery. mRNA analyses reveal that NHNP cells express a variety of phase 1 and phase 2 enzymes (Figure 3), which indicates that the cell may be capable of xenobiotic metabolism. This is important because the parent PCB congeners may be metabolized before developing toxicity (James 2001). In regard to the expression pattern of phase 1 and phase 2 enzymes, no data are available for the developing human brain. However, in adult brain, the expression of CYPs differs partially from NHNP cells (Nishimura et al. 2003); we did not identify CYP2A6 or CYP3A4 expression in NHNP cells, but adult brain exhibits a relatively high abundance of these enzymes compared with CYP1A1 expression. In contrast, neurospheres expressed CYP1A1, CYP1B1, and CYP2D6. These enzymes are also present in adult brain (Nishimura et al. 2003). Furthermore, NHNP cells express phase 2 enzymes; GSTM1 and GSTT1 were present in NHNP cells and were found in human brain tissue as well (Sherratt et al. 1997). To the contrary, human adult brain, but not NHNP cells, expressed UGT1A6 (King et al. 1999). Because of the abundance of phase 1 and phase 2 enzymes, we consider NHNP cells to be a suitable
toxicologic model for studying the effects of xenobiotics on the human developing nervous system.

TH and RA are fundamental for brain development (reviewed by Bernal et al. 2003 and by McCaffery et al. 2003). They exert their actions through nuclear hormone receptors (i.e., TR, RAR, and RXR). An important premise for investigating endocrine disruption of the thyroid hormone system by PCB is expression of the involved receptors; TRα1, β1, and β2, as well as all RAR and RXR isoforms, with exception of RARγ, were present in NHNP cells. This is in agreement with the distribution of these receptors in adult rodent brains (Zetterstrom et al. 1999). TR mRNA and protein was also detected in human fetal brain (Bernal and Pekonen 1984; Kilby et al. 2000).

In the present study, we found that the mono-ortho-substituted PCB-118, as well as TH, leads to an increased formation of oligodendrocytes in NHNP cells. The development of oligodendrocytes, which are the myelin producing cells in the central nervous system, is dependent on TH, which aids proliferation and survival of oligodendrocyte progenitor cells (Barres et al. 1994; Ben Hur et al. 1998; Schoonover et al. 2004). The importance of TH for oligodendrocyte formation was further confirmed in hypothyroid animals exhibiting fewer numbers of oligodendrocytes than control animals (Ahlgren et al. 1997).

PCBs have been observed to have an intrinsic TH-like effect: rat pups exposed to Aroclor 1254 opened their eyes at an earlier time point, an effect that is elicited with an excess of T3 (Brosvic et al. 2002; Goldey et al. 1995). In addition, in pregnant animals Aroclor treatment led to an increased expression of TH-dependent genes such as RC3/neurogranin and myelin basic protein in fetal brains (Zoeller et al. 2000), although PCB can cause a decrease of serum TH levels (Gauger et al. 2004) in vitro. Most studies performed on the effects of PCBs used Aroclor, technical mixtures of PCBs containing planar and nonplanar congeners. Because of the heterogeneity of these mixtures, we decided to apply a single congener approach with two different pentachlorobiphenyls that have weak and strong dioxin-like activities, respectively. Our results show for the first time that PCB-118 exerts a TH-like effect on a cellular level in primary human cells by increasing the number of oligodendrocytes (Figure 4).

In our study of the molecular mechanism of PCB effects on oligodendrocytes, we investigated the TH-like effect of PCB-118 and whether it is mediated through the TH receptor complex. Therefore, we performed the experiments in the presence of the specific TR antagonist NH-3. NH-3 binds to the ligand-binding domain of the TRs, with selectivity for TRβ over TRα, leading to a conformational change of the receptor with release of TR corepressors. Unlike TH, NH-3 prohibits the subsequent recruitment of TR coactivators. Specificity of TRβ inhibition was shown in vitro and in vivo (Lim et al. 2002; Nguyen et al. 2002). In the presence of NH-3 the formation of oligodendrocytes by TH and PCB-118 was blocked (Figure 5A), which may indicate that the TRβ complex is involved in PCB-118–mediated effects on oligodendrocyte differentiation. Because Gauger et al. (2004) showed that a large variety of PCBs, including PCB-118, and their metabolites do not competitively bind to TR, we speculate that the TH-like effect of PCB-118 on neural differentiation is due to facilitation of coactivator binding.

In another approach to investigate whether PCB-118 acts through the TR complex, we cotreated NHNP cells with RA. As shown in Figure 5B, RA anticipated oligodendrocyte formation induced by TH or PCB-118 treatment. RA binds to the RAR receptor, which shares its heterodimerization partner RXR with several other nuclear receptors including TR (reviewed by Rowe 1997). Therefore, we suggest that antagonism of TH or PCB-118 by RA is caused by competition over RXR. A similar antagonism of TH by RA has been described by Davis and Lazar (1992), and it has been hypothesized that participation of RXR in other activation pathways may modify the cellular response to TH (Sarlieve et al. 2004).

Regarding the metabolic capacity of these progenitor cells, we cannot exclude that the observed induction of oligodendrocytes by PCB-118 is a result of PCB metabolites rather than the parent substance, and further experiments are needed. However, the observed effect is congener specific because PCB-126 did not increase oligodendrocytes in NHNP cells. PCB-126 is a coplanar biphenyl that activates the AhR, whereas PCB-118 is mono-ortho substituted and exerts only weak AhR agonist activity (Hestermann et al. 2000). The inability of BAP, a classical AhR agonist, to induce oligodendrocyte formation in NHNP cells (data not shown) supports the suggestion that the AhR is not involved in the disturbance of neural differentiation.

In summary, we developed a primary human in vitro model for investigating endocrine disruption of neural development. We identified the mono-ortho-substituted PCB-118 as a TH disrupter on human neural development because it induced oligodendrocyte formation in NHNP cells. In contrast, PCB-126, a coplanar AhR ligand, showed no hormone-like activity. The effects seen after PCB-118 treatment seem to be mediated through the TR complex because they can be antagonized by the TR antagonist NH-3 and by RA. The precise molecular mechanisms require further elucidation.
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