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Research Article

TBBPA targets converging key events of human oligodendrocyte development resulting in two novel AOPs

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Abstract
Myelinating oligodendrocytes (OLs) establish saltatory nerve conduction during white matter development. Thus, interference with oligodendrogenesis leads to an adverse outcome on brain performance in the child due to aberrant myelination. An intertwined network of hormonal, transcriptional and biosynthetic processes regulates OLs development, thereby simultaneously creating various routes of interference for environmental toxicants. The flame retardant tetrabromobisphenol A (TBBPA) is debated as an endocrine disruptor, especially of the thyroid hormone (TH) system. We identified two independent modes-of-action (MoA) how TBBPA interferes with the establishment of a population of maturing OLs, dependent and independent of TH signaling. Combining the previously published oligodendrocyte maturation assay (NPC6) with large-scale transcriptomics, we describe TBBPA as a TH disruptor impairing human OL maturation *in vitro* by dysregulation of oligodendrogenesis-associated genes (i.e. *MBP*, *KLF9 and EGR1)*. Furthermore, TBBPA disrupts a gene expression network regulating cholesterol homeostasis, reducing OL numbers independently of TH signaling. These two MoA converge in a novel putative Adverse Outcome Pathway (AOP) network on the key event (KE) hypomyelination. Comparative analyses of human and rat neural progenitor cells (NPCs) revealed that human oligodendrogenesis is more sensitive to endocrine disruption by TBBPA. Therefore, we emphasize that ethical, cost-efficient and species-overarching *in vitro* assays are needed for DNT hazard assessment. By incorporation of large-scale transcriptomic analyses, we brought the NPC6 assay to a higher readiness level for future applications in a regulatory context. The combination of phenotypic and transcriptomic analyses helps to study MoA to eventually build AOPs for a better understanding of neurodevelopmental toxicity.

1 Introduction

Oligodendrocytes (OLs) are responsible for axon myelination thereby facilitating rapid saltatory conduction of action potentials within the central nervous system. During development, multipotent neural stem/progenitor cells (NSPCs) give rise to committed oligodendrocyte precursor cells (OPCs) which proliferate and migrate to the final site of myelination (Emery, 2010; van Tilborg et al., 2018). Subsequent terminal differentiation of OPCs into pre-myelinating OLs (pre-OLs), and finally myelin-producing mature OLs, involves a fine-tuned interplay of hormonal signaling, transcriptional regulation and biosynthetic processes. The myelin membrane contains 73% lipids, of which cholesterol makes up around 30%. Almost all brain cholesterol is synthetized *de novo*, predominantly by OLs. Strikingly, OLs synthesize more than 3-fold their own weight of myelin per day (Norton and Poduslo, 1973) making a balanced cholesterol homeostasis a necessity for oligodendrocyte maturation and axon ensheathment (Mathews and Appel, 2016; Mathews et al., 2014). Accordingly, accumulation of cholesterol due to impaired transport and export causes lipotoxicity leading to cell death of OLs (Haq et al., 2003; Bezine et al., 2017). Genetic mouse models with impaired expression of proteins regulating cholesterol clearance, such as the cholesterol exporters ABCA1 and NPC1, show reduced OPC maturation, hypomyelination and developmental delays (Wang et al., 2018; Takikita et al., 2004; Caporali et al., 2016). Consistent with that, treatment of aberrant cholesterol storage with cyclodextrin effectively restored myelination and reduced neurodegeneration in mice models of Niemann-Pick disease type C (Saher and Stumpf, 2015). In addition to a surplus of cholesterol, also the lack of cholesterol due to impaired biosynthesis causes hypomyelination in the brain. In line with this, dietary cholesterol effectively restores myelination in mouse models of myelin disorders such as Pelizaeus-Merzbacher disease (PMD) (Saher et al., 2012) and multiple sclerosis (MS) (Berghoff et al., 2017) by reestablishing OL numbers. OL maturation is further dependent on hormonal signaling.

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Especially thyroid hormones (TH) are crucial for the development of white matter tracts in humans (Annunziata et al., 1983). It is well documented that the terminal differentiation into myelinating OLs is tightly regulated by the thyroxine metabolite triiodothyronine (T3) (Baas et al., 1997; Murray and Dubois-Dalcq, 1997). Binding of T3 to nuclear thyroid hormone receptors (THRs) causes transcriptional regulation of genes under control of TH response elements (TREs) (reviewed in (Lee and Petratos, 2016)). The importance of TH signaling for OL maturation is dramatically illustrated by conditions causing hypomyelination due to TH disruption in the developing brain, such as congenital hypothyroidism (Gupta et al., 1995), maternal hypothyroidism (Wei et al., 2015), or the Allan-Herndon-Dudley syndrome (AHDS) (Gika et al., 2010; La Piana et al., 2015). Scarce TH levels during pregnancy and after birth are correlated with clinical presentations ranging from mild cognitive deficits to severe mental retardation (Rovet and Daneman, 2003; Haddow et al., 1999; Sarret et al., 1993). Importantly, TH replacement therapy has been successfully implemented in children suffering from congenital hypothyroidism (Bauer and Wassner, 2019; Gruters and Krude, 2011) and is under investigation in children with AHDS (Groeneweg et al., 2019). In accordance with the clinical observations, studies on hypothyroid rats revealed impaired expression of the myelin-associated genes myelin basic protein (*MBP*) and myelin proteolipid protein (*PLP1*) as well as reduced numbers of mature OLs (Ibarrola and Rodriguez-Pena, 1997; Schoonover et al., 2004). This is at least in part dependent on TREs present within genes associated with OL maturation such as MBP and Krüppel-like factor 9 (KLF9) (Farsetti et al., 1992; Denver and Williamson, 2009). Given the relevance of TH disruption for oligodendrocyte development, it is necessary to consider environmental exposure of the developing brain to chemicals with thyroid-disrupting potential. As a consequence, a range of environmental chemicals has been identified as TH disruptors. Modes-of-actions (MoA) include the inhibition of iodine uptake, interference with TH metabolizing enzymes, displacement of THs from transport proteins, and antagonistic or agonistic effects at the THR level (reviewed in (Zoeller, 2007)). Since differences in OL development and turnover have been described between rodents and humans, the direct translation of rodent studies into the human system is challenging (Yeung et al., 2014; Dach et al., 2017). Therefore, human cell-based *in vitro* models with a higher testing throughput than *in vivo* animal studies are needed to study the effects of chemical exposure on OL development. Our group successfully implemented the oligodendrocyte maturation assay (NPC6), which comparatively identifies disruptors of TH-dependent OL maturation in differentiating human and mouse NPC cultures (Dach et al., 2017; Bal-Price et al., 2018). Strikingly, vast species differences in OL development and resulting compound MoA were identified, emphasizing the necessity for human cell-based test systems to guarantee high predictivity for humans.

In the present study, we analyzed the effects of the brominated flame retardant (BFR) tetrabromobisphenol A (TBBPA) on OL development. TBBPA is used with high frequency in the manufacturing of household items and electronics to reduce flammability, making it a substance of high toxicological relevance in humans. Accordingly, several studies reported bioaccumulation of TBBPA in serum, cord blood, and breast milk of pregnant and nursing women (Cariou et al., 2008; Kim and Oh, 2014). Moreover, since TBBPA has a reported half-life in humans of about two days, bioaccumulation indicates chronic exposure of mothers in their daily life (Sjodin et al., 2003). Of note, increased TBBPA concentrations were found in infants compared to their mothers highlighting the particular importance of TBBPA exposure during human development.

2 Methods

2.1 Reagents

Thyroid hormone L-3,3′,5-triiodothyronine (T3, #T2877), perfluorooctanoic acid (PFOA, #171468) and 3,3′,5,5′-tetrabromobisphenyl A (TBBPA, #330396) were purchased from Merck. The following stock solutions were prepared, aliquoted, and stored at the indicated temperatures: 300 µM T3 in a 1:1 (v/v) dilution of 96% ethanol and 1 M HCl (EtOH/HCl; both Carl Roth) stored at -20 °C, 2 mM PFOA in DMSO stored at -20 °C, and 250 mM TBBPA in DMSO stored at -20 °C. The TR antagonist NH-375 (Nguyen et al., 2002; Singh et al., 2016) was diluted in DMSO and a 1 mM stock was prepared and stored at -80 °C. The final solvent concentrations within the experiments were 0.01% EtOH/HCl and 0.1% DMSO.

2.2 Neurosphere cell culture

Human neural progenitor cells (hNPCs) derived from whole-brain homogenates of male gestational week (GW) 16-19 fetuses were purchased from Lonza (#PT-2599). Time-matched rat neural progenitor cells (rNPCs) were prepared as previously described (Baumann et al., 2014). In brief, pregnant rats (Wistar) were obtained from Charles River and rNPCs were isolated from postnatal day one (PND 1) pubs by dissecting, digesting and homogenizing of whole brains to obtain a cell suspension. Brain homogenates of all pubs (male and female) were pooled prior to rNPC culture. The preparation of the rat pubs agrees with the "Landesamt für Natur, Umwelt und Verbraucherschutz" (81-02.05.50.18.001) and is according to §4 Abs. 3 Tierschutzgesetzt (TierSchG). Both hNPCs and rNPCs were cultured as 3D free-floating neurospheres in the following proliferation medium: Dulbecco's modified Eagle medium (DMEM, Thermo Fisher, #31966021) and Ham's F12 (Thermo Fisher, #31765027) were mixed 3:1 (v/v) and supplemented with B27 (Thermo Fisher, #17504044), 100U/mL penicillin and 100 µg/mL streptomycin (PAN Biotech, #P06-07100), 20 ng/mL human epidermal growth factor (EGF, Thermo Fisher, #PHG0315), and either 20 ng/mL (hNPCs) recombinant human fibroblast growth factor (FGF, R&D Systems, #233-FB) or 10 ng/ml (rNPCs) recombinant rat FGF (R&D Systems, #3339-FB-025). Both NPCs were cultured under standard cell culture conditions in a humidified incubator at 37 °C and 5 % CO_2 in cell culture dishes coated with poly (2-hydroxyethyl methacrylate) (poly-HEMA, Merck, #P3932). Once a week, the neurospheres were passaged mechanically to 0.2 mm size with a McIlwain tissue chopper (model TC752) and every second day 50% of the medium were replaced. All cultures were tested for the absence of mycoplasma and used only up to passage 3 (hNPCs) or exclusively in passage 1 (rNPCs) to guarantee high reproducibility of the test methods.

2.3 Differentiation of human and rat NPCs

In order to study the extent of oligodendrocyte maturation in response to compound exposure, human and rat NPCs were differentiated into neurons, oligodendrocytes, and astrocytes (Moors et al., 2009; Breier et al., 2010). Therefore, the following differentiation medium was prepared: DMEM (Thermo Fisher, #31966021) and Ham's F12 (Thermo Fisher, #31765027) were mixed 3:1 (v/v) and supplemented with 1% N2 (Thermo Fisher, #17502-048) and 100U/mL penicillin and 100 µg/mL streptomycin (PAN Biotech, #P06-07100). Two days prior plating, human and rat NPCs were chopped to a size of 0.2 mm in the respective proliferation media. On the plating day, 0.3 mm diameter neurospheres were sorted and washed in differentiation medium.

For the immunocytochemical staining and quantification of $O4^+$ cells, 8-chamber glass cover slides (LMS Consult) were coated with 0.1 mg/mL poly-D-lysine (PDL, Merck, #P0899) and 12.5 µg/ml laminin (Merck, #L2020). Per well, five 0.3 mm neurospheres were plated in 500 µL differentiation medium containing the respective exposure(s) or solvent control(s). The neurospheres were differentiated for 5 days in which cells radially migrated out of the sphere core (migration area). On day three of the experiment, 250 µl medium were replaced with fresh exposure/solvent medium.

For the assessment of *MBP* and *Mog* gene expression, 0.3 mm neurospheres were differentiated for 5 days in PDL/laminin coated 24-well plates (Sarstedt). Per well, 10 neurospheres were plated in 1 ml differentiation medium containing the respective exposure(s) and solvent control(s). On day three of the experiment, 500 µl medium were replaced with fresh exposure/solvent medium.

2.4 Assessment of cell viability

Cell viability was measured after 5 days of differentiation using the CellTiter-Blue (CTB) assay according to the manufacturer's instructions (Promega). Therefore, the CTB reagent was diluted 1:3 in differentiation medium and subsequently added in a ratio of 1:4 (v/v) to the 8-chamber slides. Following 2 hours of incubation under standard cell culture conditions, 2-times 100 µl supernatant per well were transferred into a 96-well plate to detect the fluorescence with a Tecan infinite M200 Pro reader (ex: 540 nm; em: 590 nm). The relative fluorescence unit (RFU) values of the replicates were averaged and medium without cells was used to correct for background fluorescence.

2.5 Immunocytochemistry

After the viability measurement, a solution of 12% paraformaldehyde (PFA, Merck) was added to the 8-chamber slides to yield a final concentration of 4% PFA. The slides were incubated at 37 °C for 45 min to fix the spheres and migrated cells. Until the immunocytochemical staining for O4, fixed slides were stored in phosphate buffered saline (PBS, Biochrom) at 4 °C. After three washing steps with PBS for 5 min, 30 µl anti-O4 antibody dilution (1:200 mouse anti-O4 (R&D Systems #MAB1326), 10% goat serum (Merck #G9023) in PBS) were added per chamber and incubated overnight at 4 °C. After three additional washing steps with PBS for 5 min, 30 µl secondary antibody solution (1:250 goat anti-mouse Alexa Fluor 488 (Thermo Fisher, #A-21042), 2% Hoechst 33258 (Merck, #B1155), 1% goat serum in PBS) were added per chamber and incubated for 30 min at 37 °C. The slides were washed three-times with PBS, once with distilled water, and finally sealed with glass cover slips using Aqua-Poly/Mount (Polysciences, #18606).

2.6 Image acquisition and quantification of $O4^+$ cells

Imaging of stained slides was performed by high content imaging analysis (HCA) using an automated fluorescence microscope (Cellomics ArrayScan VTI, Thermo Fisher Scientific).

Parameters were adjusted to detect nuclei (Hoechst, ex: 359 nm; em: 461 nm) and O4 surface expression (Alexa-488, ex: 495 nm; em: 519 nm). The image analysis was carried out with the software Omnisphero (Schmuck et al., 2017). In brief, for two defined areas (1098mm x 823mm size; once placed above and once below the sphere core) per migration area, the number of $O4^+$ cells were normalized to the total number of nuclei. The resulting percentages of the two areas from the same sphere were averaged and the mean and standard deviation was calculated for the five neurospheres of every treatment condition.

2.7 Quantitative real-time PCR

The total RNA of 5 days differentiated neurospheres was isolated and 150ng RNA were transcribed into cDNA using the RNeasy Mini Kit (Qiagen #74106) and the Quantitect Reverse Transcription Kit (Qiagen, #205313) according to manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the QuantiFast SYBR Green PCR Kit (Qiagen, #204054) within the Rotor Gene Q Cycler (Qiagen) using the primers listed in Table 1. Analysis was performed with the copy number method and *MBP*/*Mog* expression was normalized to 10.000 *ACTB* copy numbers to correct for differences in the amount of cDNA within the samples (Dach et al., 2017).

Tab. 1: Primers used for quantitative real-time PCR

Name	Primer
ACTB human_fw	5'-CAGGAAGTCCCTTGCCATCC-3'
ACTB human_rev	5'-ACCAAAAGCCTTCATACATCTCA-3'
ACTB rat_fw	5'-CCTCTATGCCAACACAGT-3'
ACTB rat_rev	5'-AGCCACCAATCCACACAG-3'
MBP human_fw	5'-CAGAGCGTCCGACTATAAATCG-3'
MBP human_rev	5'-GGTGGGTTTTCAGCGTCTA-3'
Mog rat_fw	5'-TCCATCGGACTTTTGATCCTCA-3'
Mog rat_rev	5'-CGCTCCAGGAAGACACAACC-3'

2.8 Calculation of the maturation quotient (Q_M)

As first described in (Dach et al., 2017), mature oligodendrocytes are characterized by increased *MBP* (human) or *Mog* (rat) gene expression. However, assessment of the gene expression alone is not valid, since an increase in the oligodendrocyte percentage within the NPC culture would per se lead to higher *MBP*/*Mog* levels without giving information about the maturity of the cells. Therefore, we calculated the so-called maturation quotient (Q_M), which describes the extent of *MBP*/*Mog* expression within the oligodendrocyte population. Thereby, the oligodendrocyte population is defined as the percentage of $O4^+$ cells within the differentiated NPC culture. Only, an increase in the Q_M represents an increase in oligodendrocyte maturation. Table 2 describes three possible scenarios when comparing the Q_M of exposed cells to the relevant controls.

Tab. 2: Evaluation of Q_M values

Oligodendrocyte maturation quotient (Q_M)	% $O4^+$ cells	*MBP/Mog* expression	
Q_M (exposure) > Q_M (control)	no effect	↑	increased maturation
Q_M (exposure) > Q_M (control)	↑	↑↑	increased maturation
Q_M (exposure) = Q_M (control)	↑	↑	no effect on maturation

2.9 Microarray Assays

The RNA isolation was performed using the RNeasy Mini Kit (Qiagen #74106) according to the manufacturer's protocol. Therefore, 1.000 neurospheres with a defined size of 0.1 mm were plated per well of PDL/laminin-coated 6-well-plates and cultivated for 4, 24, and 60 h for TBBPA single-treatment alone and 96 h for TBBPA/T3 co-treatment. Total RNA was quantified (Qubit RNA HS Assay, Thermo Fisher Scientific) and the quality was measured by capillary electrophoresis on a fragment analyzer and using the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples in this study showed high quality RNA Integrity Numbers (RQN; mean = 9.9).

cDNA synthesis, complementary RNA (cRNA conversion), and subsequent biotin labeling of cRNA was performed according to the manufacturer's protocol (GeneChip® WT PLUS Reagent Kit 703174 23. January 2017; ThermoFisher scientific). In brief, 100 ng of total RNA were converted to cDNA, transcribed to cRNA, followed by *in vitro* transcription and biotin labeling of cDNA. After fragmentation, labeled cDNA was hybridized to Applied Biosystems™ Clariom™ S Human Gene Expression Microarray chips for 16 hours at 45°C, stained with a streptavidin/phycoerythrin conjugate, and scanned as described in the manufacturer's protocol.

For validation of microarray analyses qRT-PCRs of a set of 10 genes were performed (Fig. S2[1]). Therefore, 500 ng RNA from microarray samples were transcribed into cDNA. RNA isolation, cDNA synthesis, and qRT-PCR were performed as described in the previous section 'Quantitative real-time PCR'.

2.10 Statistics

Experiments were performed in at least three biological replicates for each species. All data are represented as mean ± standard error of the mean (SEM). Statistical significance was calculated with two-way ANOVA and Bonferroni's post-hoc tests using GraphPad Prism 8.2.1 software. Results with p-values below 0.05 were termed significant. BMC_{30} calculation was performed by using GraphPad Prism 8.2.1 with sigmoidal curve fitting methods with therein contained BMC_{30} model.

Microarray statistical analysis

Data analyses of Affymetrix CEL files was conducted with GeneSpring GX software (Vers. 14.9.1; Agilent Technologies). To avoid inter array variability, probes were pooled by the GeneSprings' ExonRMA16 algorithm after quantile normalization of probe-level signal intensities across all samples (Bolstad et al., 2003). Input data preprocessing was concluded by baseline transformation to the median of all samples. After grouping of samples (4 biological replicates each) according to their respective experimental condition, a given probe set had to be expressed above background (i.e., fluorescence signal of that probe set was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in all 4 replicates in at least one of the conditions to be further analyzed in pairwise or ANOVA comparisons. Statistical significance was calculated with moderate t tests or one-way ANOVA. A p-value below 0.05 was considered significant. The GO terms enrichment analysis was performed using the online tool DAVID Bioinformatics Resources 6.8.

3 Results

3.1 Experimental setup for the identification of TH-dependent and -independent disruption of oligodendrocyte development

In this study, we combined the assessment of TBBPA's MoA for the development of OLs in a pre-established test method (NPC6) (Bal-Price et al., 2018; Dach et al., 2017) with large-scale gene expression analyses. Figure 1 is a schematic overview illustrating the applied test procedure. Two days prior to the assay, human (hNPCs) and rat (rNPCs) NPCs growing as neurospheres are mechanically passaged to a size of 0.2 mm diameter to ensure equal sphere sizes on the plating day and increase the reproducibility. Differentiation is initiated by transfer of 0.3 mm spheres into differentiating medium containing T3, and/or the putative TH disruptor, or solvent control. Over the following five days, approximately five percent of NPCs differentiate into O4-positive pre-myelinating OLs (pre-OLs) (Barenys et al., 2017; Schmuck et al., 2017) thereby increasing the expression of *MBP* (hNPCs) or *Mog* (rNPCs). On the fifth day of differentiation, cell viability is measured to exclude that the observed effects on pre-OL numbers or gene expression have unspecific causes. Furthermore, surface expression of O4 is detected by immunocytochemical stainings and the number of $O4^+$ cells within the differentiated NPC culture is calculated with automated high content imaging analysis (HCA)(Schmuck et al., 2017). In parallel, gene expression of *MBP* (hNPCs) or

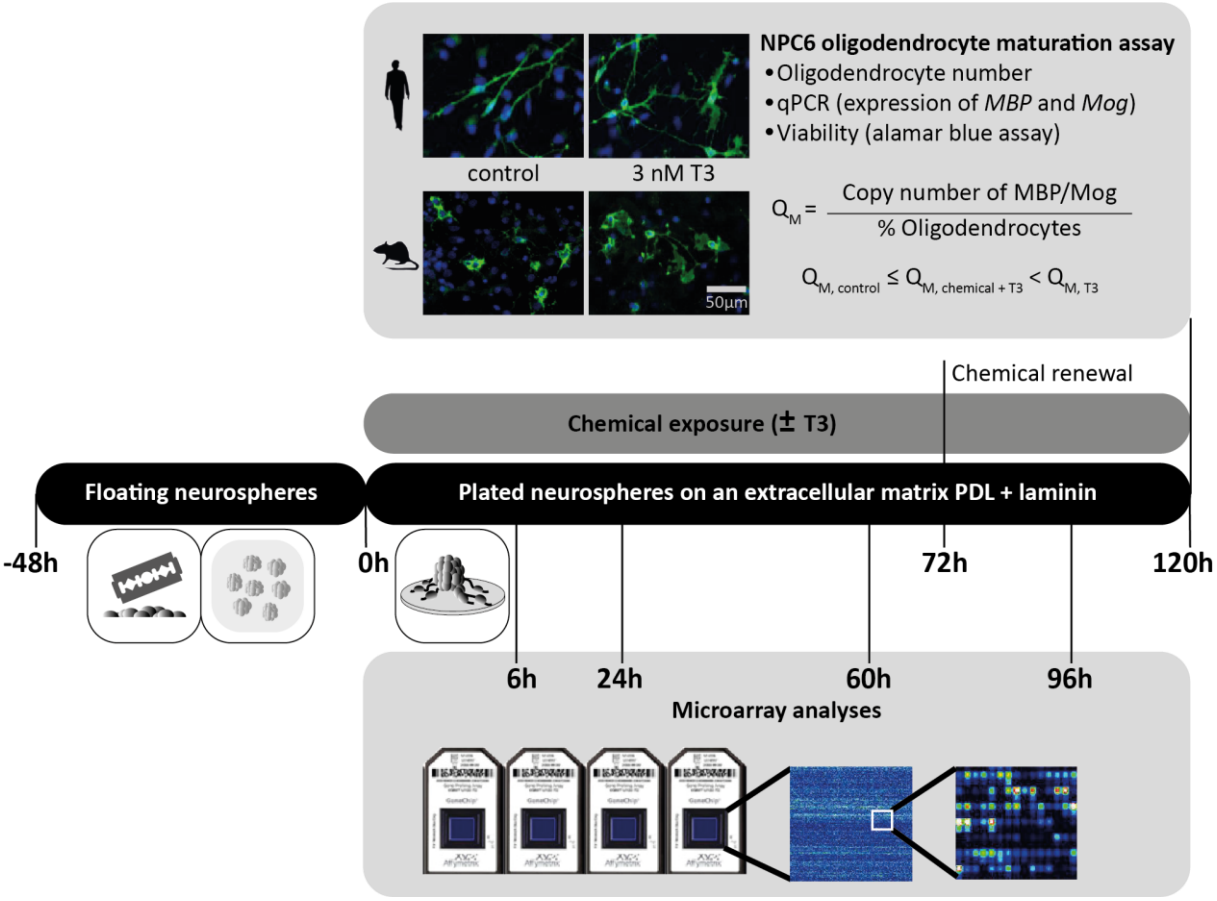

Fig. 1: Experimental setup of the oligodendrocyte maturation (NPC6) assay. Abbreviations: *MBP*, myelin basic protein; *Mog*, Myelin Oligodendrocyte Glycoprotein; PDL, Poly-D-Lysine; Q_M, maturation quotient; qPCR, quantitative real-time PCR; T3, triiodothyronine.

Mog (rNPCs) is assessed with quantitative real-time PCR (qRT-PCR). Key feature of the NPC6 assay is the maturation quotient (Q_M), which is defined as the *MBP* (hNPCs) or *Mog* (rNPCs) expression in gene copy numbers per percentage of pre-OLs within the differentiated NPC culture. The Q_M is a measure for the maturation state of the pre-OLs. T3 exposure increases the Q_M compared to the solvent control, whereas co-treatment with a TH-disruptor modulates the T3 effect and alters the Q_M (see Tab. 2). The NPC6 assay was previously established in human and mouse NPCs (Dach et al., 2017). Here, the protocol was extended to rat neurospheres (Barenys et al., 2017; Baumann et al., 2016; Masjosthusmann et al., 2018, 2019) now enabling the comparison of compound potency between primary human and rat NPC in the NPC6 assay. Furthermore, transcriptional alterations coinciding with TH-dependent and -independent effects of chemical exposure were characterized using microarray analyses of NPCs differentiating in the presence of the test compound alone (6h, 24h and 60h) or in combination with 3nM T3 (96h).

3.2 Human NPCs exhibit higher sensitivity to TH-disruption than rat NPCs

To test the performance of the NPC6 assay and study possible species-dependent effects in sensitivity to TH-disruption, a comparative analysis of OL maturation was performed with both human and rat NPCs. NPCs were differentiated for five days in the presence of 3 nM T3, increasing concentrations of the synthetic THR antagonist NH-3 which reportedly inhibits TH-dependent OL maturation (Nguyen et al., 2002; Singh et al., 2016), or a combination of both substances (Fig. 2). Cell viability was neither affected by T3, NH-3, or the combination of both substances (Fig. S1[1]). In both species, single NH-3 treatment (reddish bars) neither affected the pre-OL number, nor the *MBP*/*Mog* expression or Q_M values. On the contrary, single T3 treatment (green bars) significantly increased *MBP*/*Mog* expression and the Q_M values without affecting pre-OL numbers, confirming that T3-mediated THR signaling promotes OL maturation. T3 treatment was further accompanied by a more mature phenotype since we observed increased branching and O4-expression, as assessed with immunocytochemistry (Fig. 2B+E). When directly comparing Q_M values of the two species, OL maturation in response to T3 was thrice as pronounced in human (fold-change = 7.37) compared to rat NPCs (fold-change = 2.38; Fig. 2A+D). Co-treatment of 3 nM T3 with increasing NH-3 concentrations decreased the Q_M value in human but not in rat NPCs in a concentration-dependent manner. To further quantify the effectiveness of NH-3 as a disruptor of oligodendrocyte maturation in hNPCs and rNPCs, we calculated the bench mark concentration 30 (BMC_{30}). An NH-3 concentration of 5.4 nM reduced the Q_M by 30 % in human

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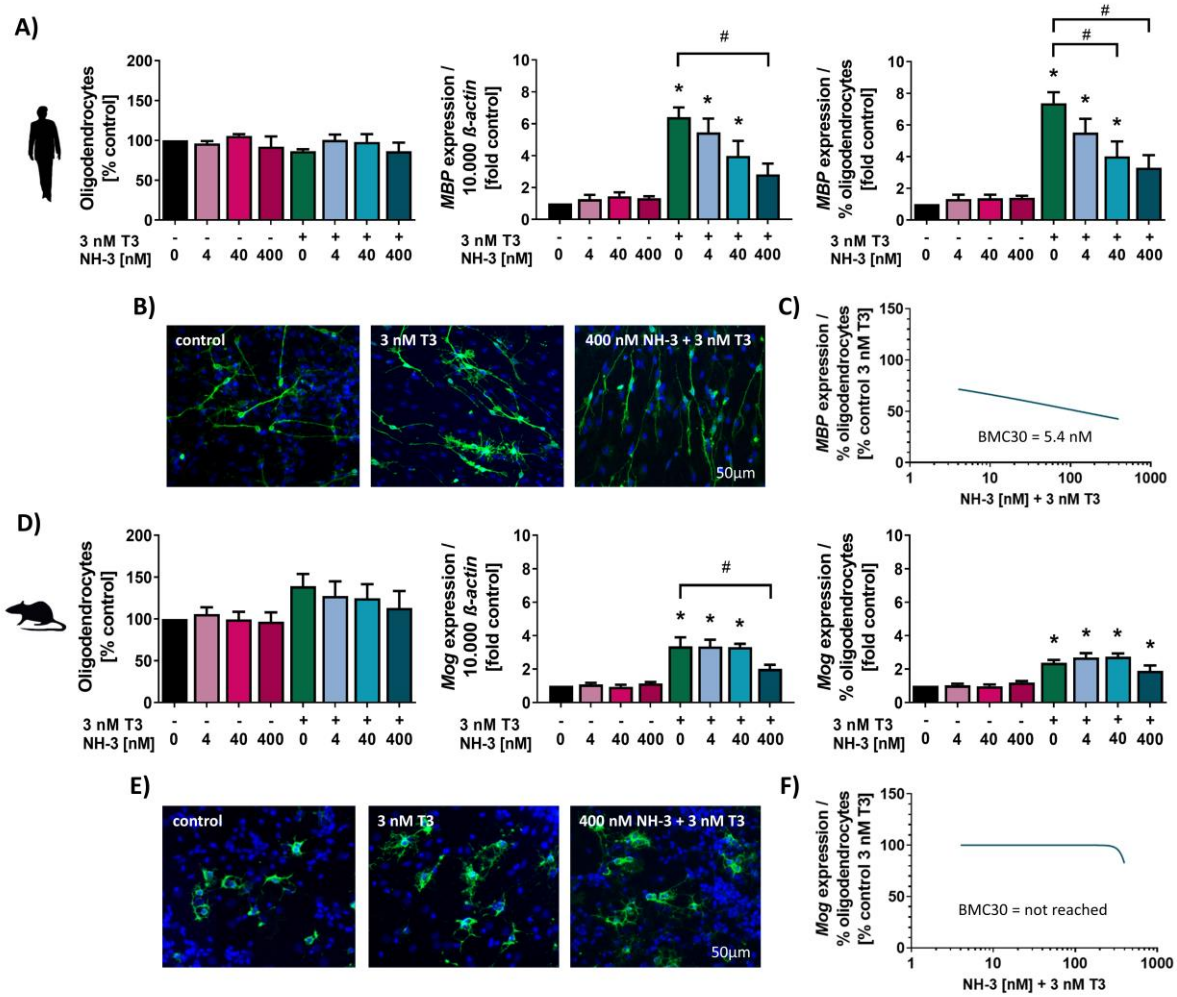

Fig. 2: Human NPCs are especially sensitive to TH-dependent disruption of OL maturation by the synthetic THR antagonist NH-3. Differentiating hNPCs (**A**) and rNPCs (**D**) were treated for 5 days with solvent (DMSO), NH-3 (4, 40, 400 nM), T3 (3 nM), or T3 in combination with increasing NH-3 concentrations. The oligodendrocyte (OL) number was defined as the percentage of O4-positive cells within the differentiated culture. *MBP* (hNPCs, A) and *Mog* (rNPCs, D) mRNA expression was assessed via RT qPCR and normalized to the housekeeping gene *ACTB*. The maturation quotient was defined as *MBP* (hNPCs, A) and *Mog* (rNPCs, D) mRNA expression per percentage of OLs. #, significant compared to the T3 single-treatment. *, significant compared to the solvent control. Representative pictures of differentiated hNPCs (**B**) and rNPCs (**E**) stained with O4 to visualize OLs. The concentration-response relationship of the TH-disruption effects of NH-3 in the co-treatment with 3nM T3 was statistically evaluated in hNPCs (**C**) and rNPCs (**F**). BMC_{30} was calculated with GraphPad Prism 8 and the sigmoidal curve fitting and BMC_{30} calculation model. At least three independent experiments (hNPCs n=3; rNPCs n=4) with 5 technical replicates were performed and represented as mean ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's post-hoc tests ($p < 0.05$ was termed significant). Abbreviations: DMSO, dimethyl sulfoxide; MBP, myelin basic protein; Mog, Myelin Oligodendrocyte Glycoprotein; T3, Triiodothyronine.

NPCs, whereas the BMC_{30} was not yet reached at NH-3 concentrations of 400 nM in rat NPCs (Fig. 2C+F). This observation demonstrates the higher sensitivity of human OLs to TH signaling and disruption.

3.3 The flame retardant TBBPA is a potent disruptor of TH-dependent oligodendrocyte maturation

Exposure to the flame retardant TBBPA was previously correlated with altered TH levels in rodents (Cope et al., 2015; Van der Ven et al., 2008) and thyroid dysfunction in humans (Mughal et al., 2018; Coperchini et al., 2017). Therefore, we studied whether TBBPA is a disruptor of TH-dependent OL maturation. Concentration finding studies revealed that after 5 days 1.7 µM TBBPA specifically reduced the number of $O4^+$ pre-OLs by 50 percent (Fig. 3A) without affecting cell viability (Fig. S1A[1]). Therefore, we limited TBBPA exposure in the NPC6 assay to concentrations not significantly affecting pre-OL numbers. In accordance with our hypothesis, TBBPA concentration-dependently reduced T3-induced OL maturation with significant effects at concentrations of 1 µM in both human and rat NPCs (Fig. 3). Similar to NH-3, TBBPA decreased the Q_M (Fig. 3B+E), the branching, and O4-staining intensity (Fig. 3C+F) of pre-OLs without affecting their numbers. However, we observed significant species differences when calculating the BMC_{30} of TBBPA in the co-treatment with T3. The BMC_{30} was almost 8-fold lower in hNPCs (0.06 µM) compared to rNPCs (0.46 µM), again emphasizing the greater sensitivity of human OLs to TH-disruption (Fig. 3D+G).

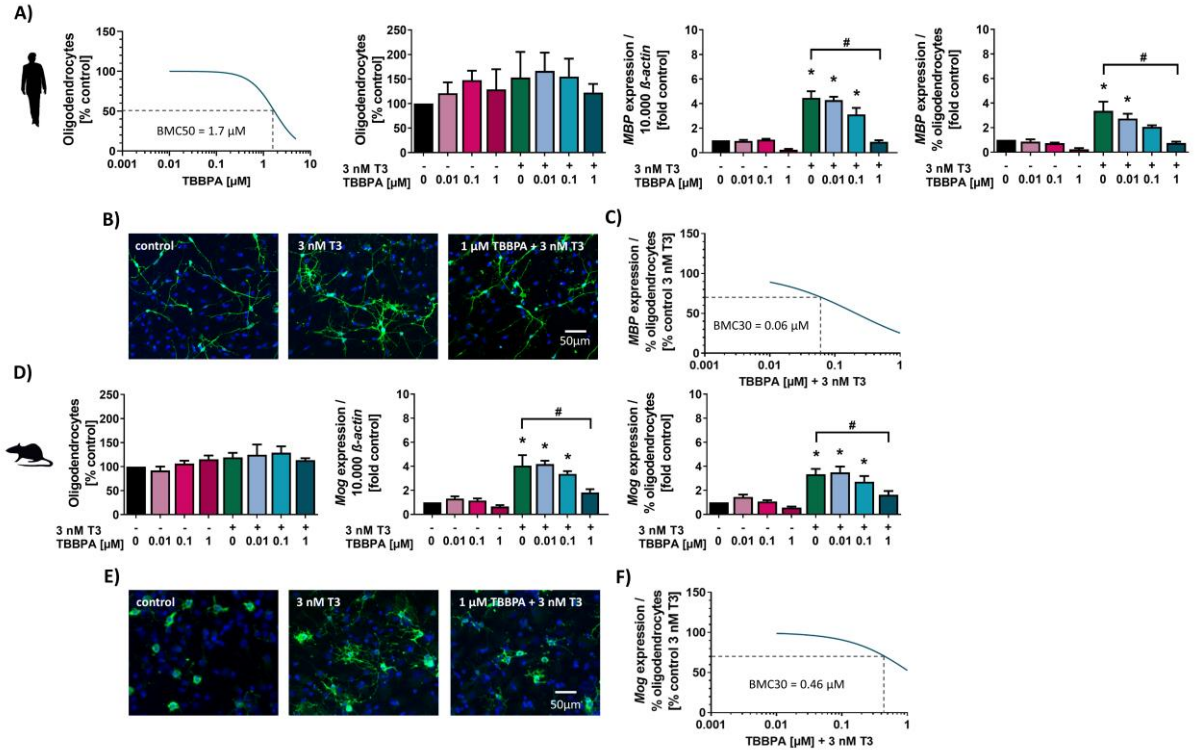

Fig. 3: TBBPA is a disruptor of TH-dependent OL maturation with higher potency in human NPCs. (**A**) Concentration-response relationship between the oligodendrocyte number and TBBPA exposure. BMC_{50} was calculated with GraphPad Prism 8 and the sigmoidal curve fitting model. Differentiating hNPCs (**B**) and rNPCs (**E**) were treated for 5 days with solvent (DMSO), TBBPA (0.01, 0.1, 1 µM), T3 (3 nM), or T3 in combination with increasing TBBPA concentrations. The oligodendrocyte (OL) number was defined as the percentage of O4-positive cells within the differentiated culture. *MBP* (hNPCs, B) and *Mog* (rNPCs, E) mRNA expression was assessed via RT qPCR and normalized to the housekeeping gene *ACTB.* The maturation quotient was defined as *MBP* (hNPCs, B) and *Mog* (rNPCs, E) mRNA expression per percentage of OLs. #, significant compared to the T3 single-treatment. *, significant compared to solvent control. Representative pictures of differentiated hNPCs (**C**) and rNPCs (**F**) stained with O4 to visualize OLs. The concentration-response relationship of the TH-disruption effects of TBBPA in the co-treatment with 3nM T3 was statistically evaluated in hNPCs (**D**) and rNPCs (**G**). BMC_{30} was calculated with GraphPad Prism 8 and the sigmoidal curve fitting and BMC_{30} calculation model. Three independent experiments with 5 technical replicates were performed and represented as mean ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's post-hoc tests ($p < 0.05$ was termed significant). Abbreviations: DMSO, dimethyl sulfoxide; MBP, myelin basic protein; Mog, Myelin Oligodendrocyte Glycoprotein; TBBPA, Tetrabromobisphenol A; T3, Triiodothyronine.

We further identified the surfactant perfluorooctanoic acid (PFOA) as a negative substance in the NPC6 assay. PFOA exposure was correlated to thyroid disfunction in humans, yet without alterations in TH levels (Lin et al., 2013; Xiao et al., 2020). In accordance with our hypothesis that the NPC6 assay detects TH-disruption on the level of THR-mediated transcription, PFOA exposure had no effect on TH-dependent OL maturation. We neither observed alterations in the Q_M values (Fig. 4A+D) nor effects on the pre-OL morphology (Fig. 4B+E) in human or rat NPCs treated with PFOA in addition to 3 nM T3. Therefore, no BMC_{30} was calculable (Fig. 4C+F). Neither TBBPA nor PFOA significantly altered NPC viability (Fig. S1B,C[1]).

We identified TBBPA, but not PFOA, as a disruptor of TH-dependent oligodendrocyte maturation with a higher potency in human cells. Furthermore, the results corroborate our hypothesis that the NPC6 assay is specific in identifying TH disruption based on impaired THR-mediated transcriptional regulation.

3.4 TBBPA disrupts TH-dependent oligodendrocyte maturation by interfering with the transcription of THR-regulated genes

Since we identified TBBPA as an endocrine disruptor in the NPC6 assay, we hypothesized that altered transcription of TH responsive genes evoke the impaired OL maturation. Microarray analysis of human NPCs differentiated for 96 h in the presence of 3 nM T3, 0.1 µM or 1 µM TBBPA, or a combination of T3 and TBBPA should reveal the strongest regulated genes responsible for the phenotypic oligodendrocyte outcome. PCA analyses indicate the highest transcriptional variation in cells treated with 3 nM T3 compared to the solvent control (dark green, Fig. 5A). Smaller variations were caused by 1 µM TBBPA single treatment (purple). The co-treatment with 3 nM T3 and 1 µM TBBPA (yellow) partially reversed the transcriptional changes induced by T3. Consistent with the phenotypic findings (Fig. 3), single and co-exposure with 0.1 µM TBBPA changed the transcriptomes only marginally. Comparing the PCA plot with the numbers of differentially regulated genes (FC ± 1.4, $p \leq 0.05$; Fig. 5B), 1 µM TBBPA (#1831) regulates more genes than 3 nM T3 (#555). However, the PCA shows that the degree of gene regulation is much higher in T3- than in TBBPA-treated hNPCs. Looking at T3-regulated transcripts affected by TBBPA (increased cut-off stringency of FC ± 2.0, $p \leq 0.05$), we identified 31 gene products as

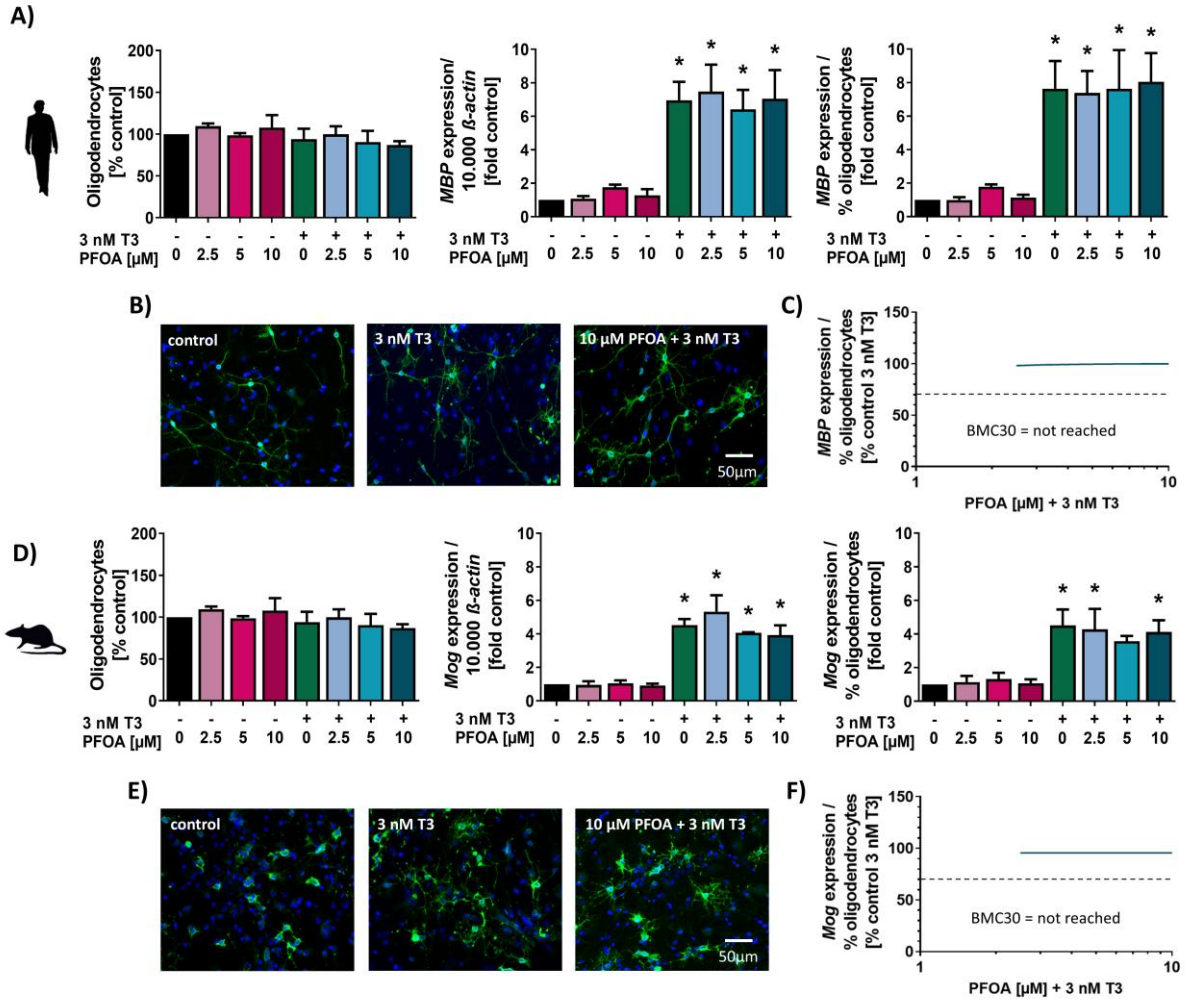

Fig. 4: PFOA is not a disruptor of TH-dependent OL maturation. Differentiating hNPCs (**A**) and rNPCs (**D**) were treated for 5 days with solvent (DMSO), PFOA (2.5, 5, 10 µM), T3 (3 nM), or T3 in combination with increasing NH-3 concentrations. The oligodendrocyte (OL) number was defined as the percentage of O4-positive cells within the differentiated culture. *MBP* (hNPCs, A) and *Mog* (rNPCs, D) mRNA expression was assessed via RT qPCR and normalized to the housekeeping gene *ACTB.* The maturation quotient was defined as *MBP* (hNPCs, **A**) and *Mog* (rNPCs, **D**) mRNA expression per percentage of OLs. #, significant compared to the T3 single-treatment. *, significant compared to the solvent control. Representative pictures of differentiated hNPCs (**B**) and rNPCs (**E**) stained with O4 to visualize OLs. The concentration-response relationship of the TH-disruption effects of PFOA in the co-treatment with 3nM T3 was statistically evaluated in hNPCs (**C**) and rNPCs (**F**). BMC_{30} was calculated with GraphPad Prism 8 and the sigmoidal curve fitting and BMC_{30} calculation model. Three independent experiments with 5 technical replicates were performed and represented as mean ± SEM. Statistical significance was calculated using two-way ANOVA and Bonferroni's post-hoc tests ($p < 0.05$ was termed significant). Abbreviations: DMSO, dimethyl sulfoxide; MBP, myelin basic protein; Mog, Myelin Oligodendrocyte Glycoprotein; PFOA, Perfluorooctanoic acid; T3, Triiodothyronine.

significantly deregulated (Fig. 5C). Additionally, we added the expression analysis of iodothyronine deiodinase 3 (*DIO3*) and Kruppel like factor 9 (*KLF9*), since we previously identified both genes to be strongly T3-dependent (Dach et al., 2017). Of these 33 genes, 23% are related to glia and oligodendrocyte development and 13% are reported TH-responsive genes. Differentiation of hNPCs under T3 exposure induced expression of the TH-responsive genes chromodomain helicase DNA binding protein 3 (*CHD3*), crystallin Mu (*CRYM*), HR lysine demethylase and nuclear receptor corepressor (*HR*), neuromedin B (*NMB*), thyroid stimulating hormone receptor (*TSHR*), *KLF9*, and *DIO3*, emphasizing the successful induction of THR signaling by T3 (Fig. 5D). As expected from a THR antagonist, 1 µM TBBPA co-exposure reduced transcription of all mentioned TH-responsive genes. Furthermore, TBBPA co-exposure altered the T3-regulated expression of six genes reportedly involved in oligodendrocyte development (early growth response 1 (*EGR1*), insulin like growth factor binding protein 4 (*IGFBP4*), interleukin 33 (*IL33*), proteolipid protein 1 (*PLP1*), serpin family E member 2 (*SERPINE2*), and *KLF9*). *EGR1*/*Krox-24* is expressed in OPCs, however, down-regulation is critical for maturation and inversely correlated with *MBP* expression (Sock et al., 1997; Topilko et al., 1997). *EGR1* expression was increased almost 2-fold in the co-treatment compared to the T3 single treatment, indicating that increased *EGR1* expression might prevent OPC maturation. We further detected that TBBPA co-exposure decreases expression of OL markers *IGFBP4* (Mewar and McMorris, 1997), *IL33* (Sung et al., 2019), *PLP1*, *SERPINE2*/*Nexin-1* (Blasi et al., 2002), and *KLF9* (Denver and Williamson, 2009) compared to the single T3 treatment, suggesting that TBBPA disturbs the transcriptome profile necessary for proper OL development. For *EGR1*, *IGFBP4*, *PLP1*, and *KLF9* TH-dependent transcription has already been reported, while *IL33* and *SERPINE2* were newly

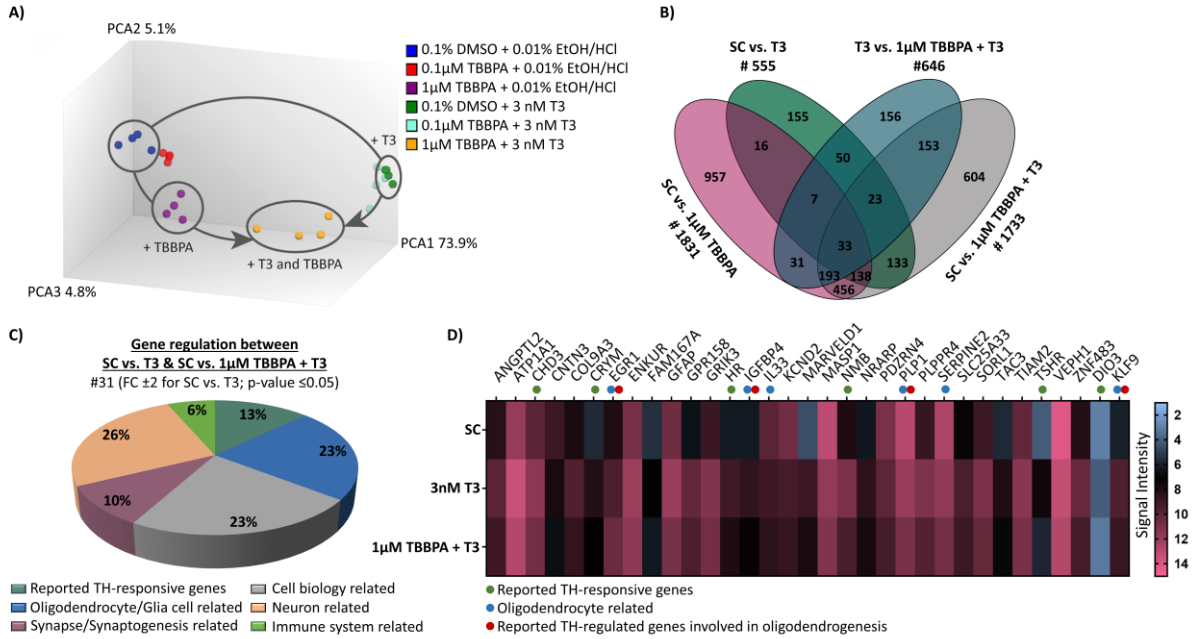

Fig. 5: Transcriptomic profiling of differentiated hNPCs revealed large-scale dysregulation of TH-responsive genes by TBBPA. Differential gene expression between hNPCs exposed to solvent (DMSO), TBBPA (0.1, 1 µM), T3 (3 nM), or TBBPA in combination with T3 was statistically determined using one-way ANOVA analyses followed by Tukey's range tests. Genes with p ≤ 0.05 and fold change ≥ 1.4 were termed differentially expressed (DEX). (**A**) Principal component analysis (PCA) was performed based on the expression of significantly regulated (p ≤ 0.05) genes that shared the same gene symbol between the above-mentioned exposure conditions. (**B**) Overlap of the number of DEX genes regulated by TBBPA (SC vs. 1 µM TBBPA, #1831), by T3 (SC vs. T3, #555), regulated T3-dependently by TBBPA (T3 vs. 1 µM TBBPA + T3, #646), and regulated by co-exposure with TBBPA and T3 (SC vs. 1 µM TBBPA + T3, #1733). (**C**) Genes differentially regulated between SC vs. T3 and SC vs. T3 + 1 µM TBBPA with an increased cut-off stringency of FC ± 2.0 and p≤ 0.05 (#31). The numbers in the pie chart represent the percentage of genes assigned to each superordinate process. (**D**) Expression profile of T3-responsive genes (FC ± 2.0 and p≤ 0.05) significantly regulated by co-exposure with TBBPA and T3. The absolute signal intensity of the 31 genes identified in (**C**), plus *DIO3* and *KLF9* as reportedly T3-responsive genes (Dach et al., 2017), was compared between hNPCs exposed to SC, T3 alone, and T3 in combination with 1 µM TBBPA. Genes are highlighted as reportedly regulated by T3 (green dots), related to oligodendrogenesis (blue dots), or associated with TH-dependent regulation of oligodendrogenesis (red dots). Abbreviations: DMSO, dimethyl sulfoxide; SC, solvent control; TBBPA, tetrabromobisphenol A; T3, triiodothyronine.

identified in this study. Thus, this data indicates that TBBPA disrupts OPC maturation by interfering with the transcription of THR-regulated genes.

3.5 TBBPA exposure disrupts oligodendrogenesis by interfering with cholesterol metabolism

Single exposure to TBBPA also affected oligodendrogenesis (BMC_{50} 1.7 µM TBBPA), yet with a TH-independent MoA, as no TH was present in the cultures during the differentiation process (Fig. 3A). To further characterize the underlying mechanisms, we performed microarray analyses of hNPCs differentiated for 6, 24, or 60 hours under exposure to either 1.7 µM TBBPA or vehicle (DMSO). Similar to observations in our previous study (Masjosthusmann et al., 2018), the PCA revealed that the highest variability in gene expression (PC1, 91.6% variation) is found over the time-course of differentiation (Fig 6A). More precisely, the 6h samples cluster further from the 24h samples than the 24h from the 60h samples, indicating that the majority of transcriptomic changes take place within the first day of differentiation compared to smaller changes during later maturation processes. The PCA further shows that less variation is caused by TBBPA exposure, however, with progressing differentiation time differences between DMSO and 1.7 µM TBBPA samples increase. To be exact, TBBPA exposure significantly ($p \leq 0.01$, fold-change ≥ 2) regulated gene expression after 6h (16 genes), 24h (11 genes), and 60h (34 genes) of NPC differentiation (Fig. 6B). Only two genes were regulated by TBBPA throughout the whole differentiation process: Expressions of *TXNIP* (thioredoxin interacting protein) and the cholesterol exporter *ABCA1* (ATP-binding cassette (ABC) subfamily-A transporter 1) were reduced by TBBPA exposure after 6 h, 24 h, and 60 h of differentiation (Fig 6C). Yet, of the 51 differentially expressed genes, we identified 16 (31%) to be involved in cholesterol metabolism (blue dot, Fig. 6C). For 10 of these genes, a correlation with OL differentiation or myelination has already been described (blue and red dots, Fig. 6C). In accordance with that, gene ontology enrichment analysis revealed a high proportion of GO terms involved in cholesterol metabolism (blue) after 24h (Fig. 6D) and 60h (Fig. 6E+F) TBBPA exposure. Therefore, we postulate that interference of TBBPA with cholesterol metabolism is a second, TH-independent MoA for disruption of oligodendrogenesis. How TBBPA interferes with genes involved in cholesterol metabolism, i.e. the molecular initiating event (MIE), remains elusive.

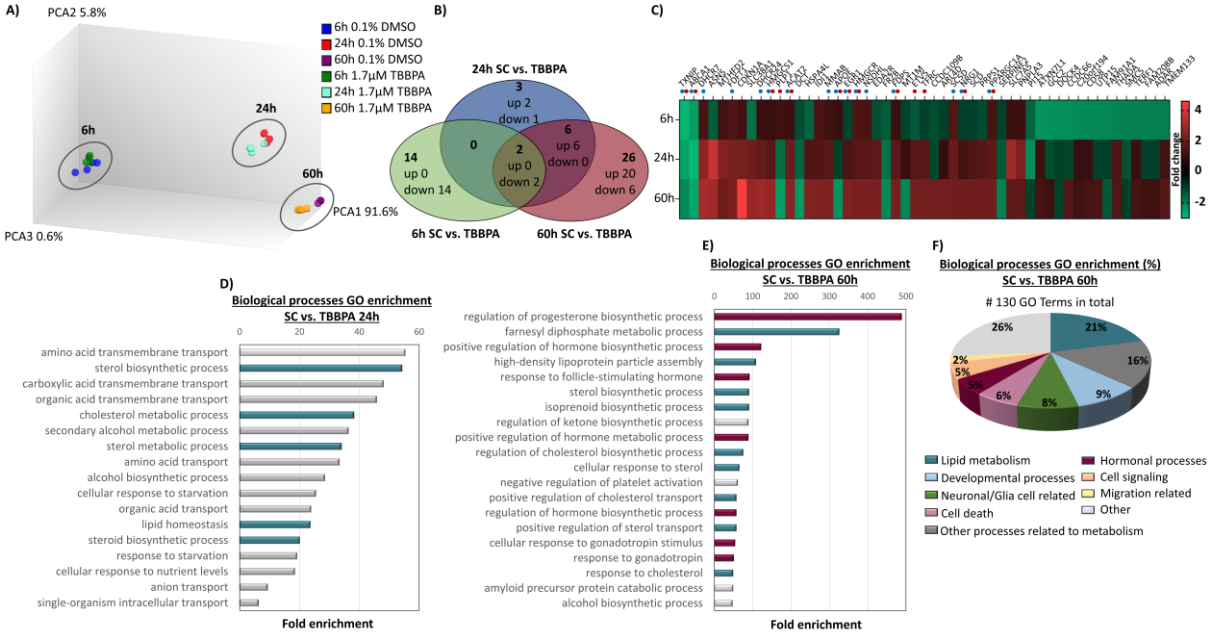

Fig. 6: TBBPA deregulates a gene cluster involved in cholesterol metabolism independently of TH-signaling. Differential gene expression between hNPCs exposed to solvent (DMSO) or TBBPA (0.1, 1 µM) over the time-course (6, 24, and 60h) of differentiation was statistically determined using one-way ANOVA analyses followed by Tukey's range tests. Genes with $p \leq 0.01$ and fold change ≥ 2 were termed differentially expressed (DEX). **A.** PCA was performed based on the expression of significantly regulated ($p \leq 0.01$) genes between the above-mentioned exposure conditions. **B.** Overlap of the number of DEX genes regulated by 1 µM TBBPA over the time-course of 6h (6h SC vs. 1 µM TBBPA, #16), 24h (24h SC vs. 1 µM TBBPA, #11), and 60h (60h SC vs. 1 µM TBBPA, #34) of hNPC differentiation. **C.** The expression profile of differentially regulated genes (fold-change) between SC and 1 µM TBBPA over the time-course (6, 24, and 60h) of hNPC differentiation. **D+E.** Overrepresented gene ontology (GO) terms for 24h (D) and 60h (E) differentiation of hNPC under 1 µM TBBPA exposure. GO enrichment analysis was performed using the online tool DAVID Bioinformatics Resources 6.8. **F.** All overrepresented GO terms were sorted by their fold enrichment and displayed are the GO terms with highest fold-enrichment. The GO terms after 60h of differentiation were further assigned to 9 superordinate processes based on expert judgment. Numbers in the pie chart represent the percentage of GO terms assigned to each superordinate process. Abbreviations: DMSO, dimethyl sulfoxide; SC, solvent control; TBBPA, tetrabromobisphenol A; T3, triiodothyronine.

3.6 Two putative AOPs for the disruption of OL development converge in hypomyelination

Combining the NPC6 assay with microarray analysis, we identified two putative MoA how TBBPA interferes with OL development. The first involves TH disruption, most likely via displacing T3 from the THR and dysregulation of THR-dependent transcription (Fig. 7A). Here we hypothesize that dysregulation of genes necessary for OL maturation, such as *EGR1/Krox-24*, *IL-33*, and *KLF-9*, cause the less mature state we observed in pre-OLs differentiated in the presence of 1 µM TBBPA. The second MoA involves the dysregulation of a whole gene expression network involved in cholesterol metabolism (Fig. 7B). Since effective cholesterol synthesis and clearance is a prerequisite for OPC survival, TBBPA exposure might reduce pre-OL numbers by interfering with early stages of OL development. These observations lead us to the drafting of two converging putative Adverse Outcome Pathways (AOPs) depicted in figure 7C. The **Thyroid Hormone Disruption AOP** starts with the competitive binding of a chemical to the THR (MIE) which deregulates genes necessary for OL maturation and thus reduces the maturation state of the pre-OLs. The **Altered Cholesterol Metabolism AOP** starts with an, until now, unknown MIE which leads to the dysregulation of genes involved in cholesterol metabolism. The disturbed cholesterol homeostasis impairs the survival of OPCs thereby reducing the number of pre-OLs. Finally, both AOPs converge in a key event (KE) during brain development where less myelin-producing OLs are present. This causes alterations in the white matter leading to adverse outcomes such as cognitive, attentional, behavioral, and social deficits in the developing child.

4 Discussion

Oligodendrocytes are indispensable for normal brain development and function (Barateiro et al., 2016). Hence, disturbed oligodendrogenesis and resulting hypomyelination cause a functional adverse outcome on brain performance manifesting in neurological disorders such as AHDS (Sarret et al., 1993) and periventricular leukomalacia (PVL) (Back et al., 2001). Since the oligodendrocyte population established in childhood remains remarkably stable in humans, neurodevelopmental interference in oligodendrogenesis is especially problematic (Yeung et al., 2014). Oligodendrogenesis is regulated through a variety of hormonal, transcriptional and biosynthetic processes, creating various routes of interference for environmental toxicants. Since OL development begins during fetal development and continuous throughout the first two years of life, exposure of pregnant women and children to compounds interfering with OL development has a high potential of causing an adverse outcome for brain development. In the present study, we identified the flame retardant TBBPA as a potent disruptor

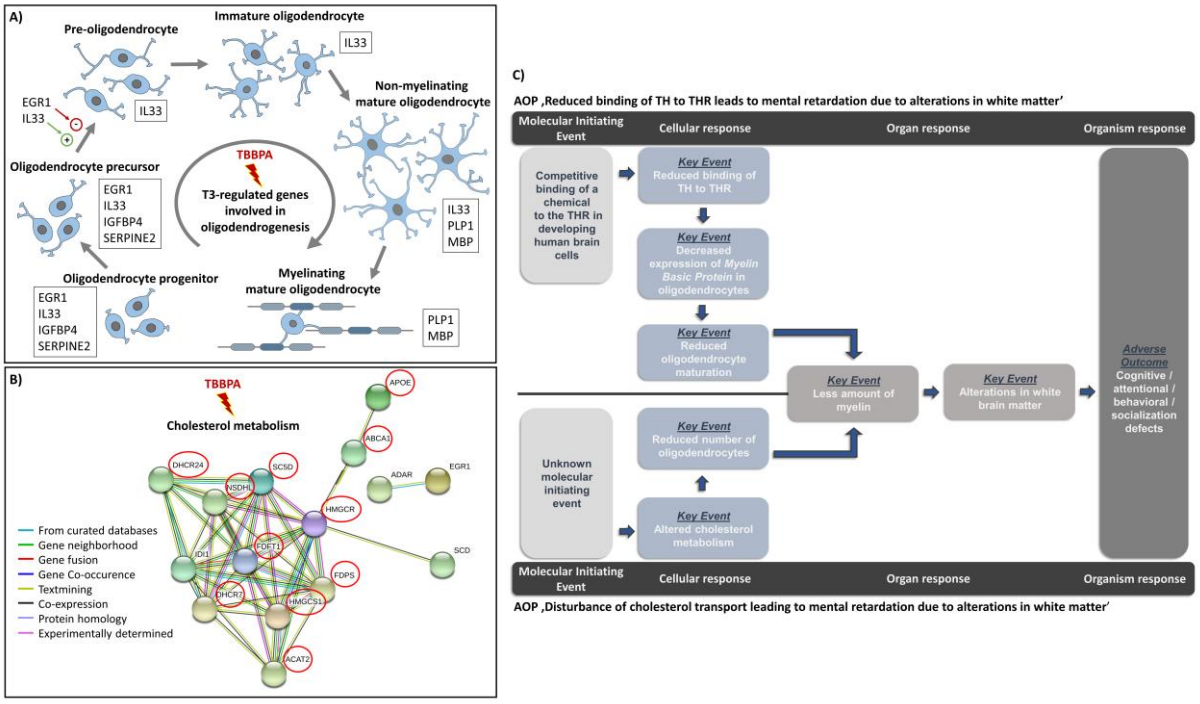

Fig. 7: Putative schematic AOPs for the converging key events in OL development disrupted by TBBPA. A. Genes TH-dependently regulated by 1 µM TBBPA which are reportedly involved in oligodendrogenesis. **B.** Gene-gene interaction network analysis of the genes regulated TH-independently by 1.7 µM TBPPA show involvement of cholesterol metabolism. **C.** The putative "Thyroid Hormone Disruption AOP" includes the TH-dependent disruption of OL maturation as a central key event. The "Altered cholesterol metabolism AOP" comprises reduced OL numbers due to TH-independent dysregulation of cholesterol metabolism as a key event. Both AOPs converge in the common key event "less amount of myelin" resulting in white matter alterations and adverse outcomes in the developing brain.

of oligodendrogenesis. TBBPA is intensively used in the manufacturing of consumer products and therefore reported as a ubiquitous environmental and indoor contaminant (Matsukami et al., 2015; Ni and Zeng, 2013; Yang et al., 2012). It was detected at a high frequency in maternal serum, cord blood serum, and breast milk samples (Abdallah and Harrad, 2011; Cariou et al., 2008; Kim and Oh, 2014) with average contamination levels in breast milk increasing steadily over the years (Shi et al., 2017). In adults, the bioavailability of TBBPA is relatively low since it is efficiently metabolized and excreted (Schauer et al., 2006). Measurements in cord blood, however, show its presence in the fetal circulation. Since the gastrointestinal tract and the metabolic capacity of fetal and infant liver are not fully developed (Veereman-Wauters, 1996; Coughtrie et al., 1988), TBBPA exposure results in bioaccumulation in the developing child *in utero* (Cariou et al., 2008; Kim and Oh, 2014).

TBBPA has frequently been discussed concerning its potency as an endocrine disruptor, especially of the TH system (Mughal et al., 2018; Coperchini et al., 2017). TBBPA impeded the binding of T3 to the THR (Kitamura et al., 2002, 2005) and enhanced the proliferation of the rat pituitary reporter cell line GH-3 *in vitro* (Kitamura et al., 2002; Hamers et al., 2006). Since thyroid hormones are indispensable for fetal development (Patel et al., 2011; de Escobar et al., 2004), TBBPA-mediated TH disruption has been studied under a developmental context. *In vivo* studies in *Danio rerio* (zebrafish) (Zhu et al., 2018; Parsons et al., 2019) and *Xenopus laevis* (Wang et al., 2017; Zhang et al., 2014) showed that developmental exposure to TBBPA reduced expression of THR-regulated genes (*THRB* and *DIO3*) and inhibited T3-induced morphological changes in the nanomolar range. Moreover, reproductive toxicity studies in rats revealed altered serum T3 concentrations (Saegusa et al., 2009; Van der Ven et al., 2008) and decreased circulating T4 (Cope et al., 2015; Van der Ven et al., 2008) after developmental exposure of the offspring. Yet so far, the adverse effects of TBBPA on human TH-dependent neurodevelopment have been enigmatic. Here we report for the first time that TBBPA effectively disturbs T3-mediated THR signaling in developing primary human NPCs by i) altering their TH-dependent gene expression and ii) by antagonizing TH-mediated OL maturation.

T3 exposure regulated several genes during hNPC development. These include genes that were previously reported to be TH-regulated and contribute to oligodendrogenesis, i.e. *KLF9, PLP1, IGFBP4 and EGR1* as well as genes that were newly identified as T3-regulated in this study (*IL33* and *SERPINE2*). The transcription factor *KLF9* for example is induced in the mouse hippocampus and cerebellum (Denver and Williamson, 2009), rat cortex and the zebrafish embryo (Walter et al., 2019) during development. In addition, T3 induces *KLF9* expression in rat OPCs *in vitro* (Dugas et al., 2012) suggesting its role in oligodendrogenesis and/or myelin production. This is supported by *in vivo* studies reporting that KLF9 is necessary for T3-induced zebrafish OL maturation and MBP/*Mog* expression (Dugas et al., 2012). In contrast, KLF9$^{-/-}$ mice do not show delayed OL differentiation but delayed myelin regeneration in cortex and corpus callosum (Dugas et al., 2012). However, since TH-dependent OL differentiation and maturation are regulated in a species-specific way (Dach et al., 2017), this is not surprising. Here we show that co-exposure of developing human NPC with T3/TBBPA antagonizes T3-induced *KLF9* expression supporting the phenotypic observation that TBBPA decreased T3-dependent human OL maturation.

Whether the TBBPA-antagonized T3-dependent *MBP* expression in hNPCs happens directly or is mediated through KLF9 is not known and could be subject to further investigation. Besides impaired KLF9 signaling, the increased *EGR1*/*Krox-24* expression we detected upon T3/TBBPA co-exposure might also contribute to the disturbed OL maturation since down-regulation of EGR1 is critical for OPC differentiation into a mature myelinating state (Sock et al., 1997; Topilko et al., 1997) and coincides with increased MBP expression during OL development in rat (Sock et al., 1997). In contrast to the negative regulation of *EGR1*, up-regulation of *IL33* is necessary for OL lineage progression (Sung et al., 2019). In this study, IL33 expression is reduced by T3 and further down-regulated in the T3/TBBPA co-exposure indicating its involvement in the decelerated OL maturation. Of note, mice lacking IL33 exhibit several behavioral deficits (Dohi et al., 2017). Besides markers regulating OL lineage progression, co-exposure with TBBPA further reduced the expression of T3-impaired *PLP1*, the main component of myelin produced by mature OLs. Consistent with our observations, transcriptomic analyses of TBBPA-exposed zebrafish embryos identified oligodendrocyte development as the most sensitive GO term (Chen et al., 2016) and confirmed suppression of *MBP* (Zhu et al., 2018). Of note, thyroid response elements are present within the promoters of *KLF9* (Denver and Williamson, 2009), *EGR1* (Ghorbel et al., 1999), and *MBP* (Farsetti et al., 1992), suggesting that the observed TBBPA-effect is at least to some extent dependent on TH disruption on the receptor level. While most studies report TBBPA as a strong T3-antagonist, also agonistic effects were previously reported in *in vitro* studies (Hamers et al., 2006; Kitamura et al., 2002). Why TBBPA in this study produces TH-antagonistic and -mimetic effects on TH-regulated genes in the same cell system remains puzzling. In summary, these data strongly support the notion that TBBPA interferes with TH signaling of primary human NPC.

On a cellular level, we demonstrate that TBBPA acts as a THR antagonist by reducing the T3-dependent maturation of human OL *in vitro*. Disruption of THR signaling has been reported to impair oligodendrogenesis leading to adverse outcomes in brain development (Baas et al., 1997; Billon et al., 2002). In accordance, OL dysfunction causes diverse behavioral and cognitive deficits (Kawamura et al., 2020; Back et al., 2001). That TBBPA exposure impairs normal brain function through interference with neurodevelopmental processes was reported in *in vivo* studies earlier. Developmental TBBPA exposure of rodents increased anxiety-related behavior (Rock et al., 2019), reduced the sociability (Kim et al., 2015) and impaired the auditory response (Lilienthal et al., 2008). Moreover, TBBPA concentrations in the nanomolar range caused hypoactivity in zebrafish embryos both dependent (Zhu et al., 2018) and independent (Noyes et al., 2015) of TH-signaling. We therefore hypothesize that TBBPA causes behavioral and cognitive defects at least in part via disruption of TH-dependent OL maturation. By comparative testing in human and rat NPCs we elucidated species differences in sensitivity to TH-disruption. Human NPCs were more sensitive to T3-dependent disruption of OL maturation both by the synthetic THR antagonist NH-3 (BMC_{30} = 5.4 nM in hNPCs, not reached in rNPCs) and the flame retardant TBBPA (7.7-fold lower in hNPCs). This clearly illustrates the need for test systems based on human cells in order to achieve adequate predictivity of hazard for humans. TBBPA accumulated in the mouse striatum at acute exposure doses as low as 0.1 mg/kg/d to measured brain concentrations of 0.1 nM which caused anxiety-related behavioral effects (Nakajima et al., 2009). Moreover, behavioral studies in Zebrafish detected hypoactivity after developmental exposure to 6.4 nM TBBPA (Noyes et al., 2015) and studies in Xenopus revealed efficient disruption of TH-induced morphological changes after exposure of tadpoles to 10 nM TBBPA (Zhang et al., 2014; Wang et al., 2017). These *in vivo* doses are below the range of the TH-disrupting effect of TBBPA observed in this study. However, it is difficult to compare a compound's tissue levels with nominal medium concentrations. For a direct comparison of *in vivo* with *in vitro* potency, IVIVE (*in vitro*-*in vivo* extrapolation) has to be performed (Wetmore et al., 2012; Bell et al., 2017). Human exposure studies measured TBBPA concentrations of up to 3.02 nM in breast milk leading to an estimated daily intake of up to 1.314 µg TBBPA by breast feeding alone. Additionally, the combined exposure of the infant to TBBPA via breast milk and house dust has to be considered (Malliari and Kalantzi, 2017). Amongst the highest TBBPA exposure levels are house dust samples from Japanese homes with 490-520 ng TBBPA per gram dust (Takigami et al., 2009). Furthermore, the frequent exposure to mixtures of several persistent organic pollutants may potentiate adverse effects (Lenters et al., 2019; Braun et al., 2020). In conclusion, the increased sensitivity of the human compared to rodent neurodevelopment together with the developmental adverse effects upon nanomolar exposure observed in *in vivo* studies, indicate a potential risk of TBBPA in the neurodevelopmental context. Kinetic evaluation including IVIVE, physiologically based toxicokinetic (PBTK) modeling, and biokinetics of intracellular TBBPA distribution within the culture systems are needed for a more precise risk characterization. Since the sex evidently affects oligodendrocyte development (Yasuda et al., 2020) and the exposure response to TH disrupting chemicals (Leonetti et al., 2016; Przybyla et al., 2018), the use of exclusively male hNPCs is a limitation of this study. By integrating female hNPCs into the NPC6 assay in the future, we will be able to detect both sex- and species-specific effects.

Besides its action as an endocrine disruptor, we observed that TBBPA reduces the number of pre-OLs independent of TH-disruption at similar concentrations. Upon single TBBPA exposure, we observed several gene product for enzymes of cholesterol biosynthesis (7-dehydrocholesterol reductase (*DHCR7*), 24-dehydrocholesterol reductase (*DHCR24*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*), farnesyl diphosphate synthase (*FDPS*)) being upregulated (Fig. 6), including the mRNA for the rate-limiting enzyme hydroxymethylglutaryl-CoA synthase (*HMGCS1*). The high rate of cholesterol utilization in myelin-producing OLs (Norton and Poduslo, 1973) emphasizes the necessity to tightly regulate cholesterol homeostasis, since both insufficient and excessive cholesterol levels are associated with brain pathologies. Under physiological conditions, excessive intracellular cholesterol is bound by the main cholesterol transport protein apoE and exported out of the cell by ABCA1. However, TBBPA significantly reduced the gene expression of apoE (*APOE*) and *ABCA1* in differentiating hNPC (Fig. 6). This suggests that cholesterol is less efficiently transported by apoE and ABCA1 causing a positive feedback of cholesterol biosynthesis with subsequent accumulation in the developing OLs. The importance of cholesterol clearance is elucidated by studies on ABCA1- and apoE-knockout mice. Disturbed cholesterol export in $ABCA1^{-/-}$ mice reduced the proliferation and survival of OPCs and impaired myelination in the corpus callosum (Wang et al., 2018), whereas increased apoptosis of OLs was detected in $apoE^{-/-}$ mice (Cheng et al., 2018). Moreover, knockdown of apoE in neural stem cells (NSCs) dramatically decreased the number of $O4^{+}$ OLs, a phenotype which was efficiently rescued by apoE protein administration (Gan et al., 2011). The importance of

cholesterol transport and export for OL survival is further elucidated by several studies reporting that induced expression or administration of ABCA1 and apoE increases OPC numbers (Cui et al., 2017; Safina et al., 2016; Wang et al., 2018), improves myelination (Zhou et al., 2019; Stoll et al., 1989), and enhances cholesterol efflux (Nelissen et al., 2012). Furthermore, $apoE^{-/-}$ mice fed with a high-cholesterol diet exhibit lipotoxicity as a result of impaired cholesterol clearance (Aung et al., 2016). Lipotoxicity was also correlated with accumulation of biosynthetic precursors and break-down products in oligodendrocytes (Haq et al., 2003; Bezine et al., 2017). Hence, we propose that TBBPA, independent of TH-disruption, decreases the number of pre-OLs by interfering with cholesterol homeostasis. However, further studies are needed to elucidate how exactly TBBPA deregulates THR-independent gene expression.

Since OLs represent only approximately 5% of the total differentiated hNPC culture, possible effects of TBBPA on the other cell types (NSCs, radial glia, astrocytes, and neurons) cannot be neglected. Therefore, as part of a comprehensive study on 15 widely used flame retardants (Klose et al., manuscript in preparation) we screened TBBPA in our established hNPC-based DNT battery with NPC proliferation and viability, radial glia migration, oligodendrocyte differentiation and migration, as well as neuronal differentiation and migration as phenotypical readouts (Nimtz et al., 2019). Strikingly, we identified impaired OL differentiation/number as the most sensitive DNT effect caused by TBBPA exposure across all studied endpoints. TBBPA impairs rat NCS (>25 µM (Cho et al., 2020)) and human embryonic stem cell-derived NCS (>100 µM (Liang et al., 2019)) survival at concentrations we did not test (Klose et al., manuscript in preparation) as they are most likely not relevant for human exposure. Besides OLs, proper regulation of cholesterol homeostasis is crucial for neurons and astrocytes (Pfrieger and Ungerer, 2011) and disturbances are associated with disease (Valenza et al., 2015). Neither neuronal differentiation nor their migration were specifically affected by up to 20 µM TBBPA treatment (Klose et al., manuscript in preparation) indicating that these processes are less sensitive towards cholesterol homeostasis disturbances than cells of the OL lineage. However, transcriptional changes displayed by the microarray analyses of the mixed culture could originate from other cell types besides OLs. For example, KLF9 and EGR1, are crucial for neuronal maturation and synaptic plasticity, respectively (Scobie et al., 2009; Duclot and Kabbaj, 2017). These endpoints are not reflected in this neurosphere *in vitro* system due to their later stage nature supporting the concept that not only the different cell types but also a variety of neurodevelopmental processes reflecting distinct developmental timing is crucial to be reflected in DNT *in vitro* assessment. Concerning astrocytes, we cannot exclude effects on this cell type since they are not yet covered by the hNPC DNT battery.

Interruption of cholesterol biosynthesis in a DNT context was recently studied in a ToxCast™ chemical library screening effort using mouse neuroblastoma Neuro-2a cells with subsequent validation in human induced pluripotent stem cell (hiPSC)-derived NPC (Wages et al., 2020). TBBPA did not present itself as one of the lead-hits possibly due to cell type-specificity of effects since OLs were not included in the ToxCast™ study, yet seem to be the most sensitive cells in the neurosphere mixed culture for this MoA (data not shown). Studying the lead-hits for disturbance of cholesterol synthesis identified by Wages et al. (2020) in the NPC assays (Bal-Price et al., 2018) would shed more light on cell types most sensitive for this MoA. This instance demonstrates that DNT MoA can be cell type-specific. When *in vitro* assays are used for DNT hazard assessment, a broad variety of brain cell types and neurodevelopmental processes should be used to identify the largest variety of MoA and reduce uncertainty, especially of negative results.

These two identified MoA for disturbance of oligodendrocyte development, i.e. TH-disruption and disturbance of cholesterol transport, converge in an AOP network on the KE myelin production (Fig. 7). The AOP 'Reduced binding of TH to THR in developing brain cells leads to mental retardation due to alterations in white matter' was already submitted to the OECD based on our previous work (Dach et al., 2017). It converges with the recently published AOP network for chemically-induced thyroid activity (Noyes et al., 2019) at the level of the MIE 'TRα binding' in developing brain cells. That TRα and not TRβ is involved in this AOP was hypothesized earlier and at this point builds on data translated from mice and of the approximately 30-fold higher TRα than TRβ mRNA expression in the developing hNPC (Dach et al., 2017). In contrast, the so far unpublished AOP 'Disturbance of cholesterol transport leading to mental retardation due to alterations in white matter' is based on the current novel observations that TBBPA deregulates gene expression of a gene cluster concerning cholesterol biosynthesis and transport coinciding with OL toxicity. More compounds acting via these MoA as well as rescue experiments are needed to strengthen these two hypothetical AOPs by experimentally establishing KE relationships. Increasing the number of and converging KE for DNT will eventually lead to a large AOP network facilitating the interpretation of *in vitro* assays for the multiple applications within a risk assessment framework for DNT (Bal-Price et al., 2015). Due to the complexity of gene regulation, we cannot exclude that some TH-responsive genes are additionally regulated TH-independently by TBBPA. However, the strong antagonistic effect of TBBPA on the TH-dependent expression of known TH-responsive genes involved in OL maturation such as *KLF9* highlights its efficacy as a TH signaling disrupter during oligodendrogenesis.

In summary, we identified two MoA how TBBPA interferes with the establishment of a population of mature, myelin-producing OLs necessary for white matter development. Since our comparative analyses revealed a higher sensitivity of human compared to rat NPC, we want to point out that ethical and cost-efficient *in vitro* assays based on human cells are needed to identify chemicals with the potential to disrupt oligodendrogenesis. In this study, we brought the previously published oligodendrocyte maturation assay (NPC6) (Bal-Price et al., 2018; Dach et al., 2017) to a higher readiness level for future applications in a regulatory context.

This study demonstrates that species-overarching neurosphere assays are well suited for hazard assessment of DNT and that phenotypic combined with transcriptome analyses are powerful tools for understanding MoA and eventually building AOPs for a better comprehension of the DNT hazard. Especially the multi-cellularity of the neurosphere assay, presenting NPC, radial glia, neurons, astrocytes and oligodendrocytes makes this test method an effective instrument for multiple MoA discovery which is urgently needed for a broader and more in-depth understanding of neurodevelopmental toxicity.

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Conflict of interest

The authors declare no conflicts of interest.

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