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Risk assessment of parabens in a transcriptomics-based in vitro test

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ABSTRACT

Parabens have been used for decades as preservatives in food, drugs and cosmetics. The majority however, were banned in 2009 and 2014 leaving only methyl-, ethyl-, propyl-, and butyl-derivates available for subsequent use. Methyl- and propylparaben have been extensively tested in vivo, with no resulting evidence for developmental and reproductive toxicity (DART). In contrast, ethylparaben has not yet been tested for DART in animal experiments, and it is currently debated if additional animal studies are warranted. In order to perform a comparison of the four currently approved parabens, we used a previously established in vitro test based on human induced pluripotent stem cells (iPSC) that are exposed to test substances during their differentiation to neuroectodermal cells. EC_{50} values for cytotoxicity were 906 μM, 698 μM, 216 μM and 63 μM for methyl-, ethyl-, propyl- and butylparaben, respectively, demonstrating that cytotoxicity increases with increasing alkyl chain length. Genome-wide analysis demonstrated that FDR-adjusted significant gene expression changes occurred only at cytotoxic or close to cytotoxic concentrations, for example 1720 differentially expressed genes (DEG) at 1000 μM ethylparaben, 1 DEG at 316 μM, and no DEG at 100 μM or lower concentrations. The highest concentration of ethylparaben that did not induce any cytotoxicity nor DEG was 1670-fold above the highest concentration reported in biomonitoring studies (60 nM ethylparaben in cord blood). In conclusion, cytotoxicity and gene expression alterations of ethylparaben occurred at concentrations of approximately three orders of magnitude above human blood concentrations; moreover, the substance fitted well into a scenario where toxicity increases with the alkyl chain length, and gene expression changes only occur at cytotoxic or close to cytotoxic concentrations. Therefore, no evidence was obtained suggesting that in vivo DART with ethylparaben would lead to different results as the methyl- or propyl derivates.

1. Introduction

Parahydroxybenzoates, often named 'parabens', are esters of 4-hydroxybenzoic acid and are effective antimicrobials and antifungals - characteristics that have supported their use as preservatives in the food, drugs and cosmetics industry for more than 70 years [1,2]. Currently, their continued use is critically discussed as parabens may compromise human health, especially as potential endocrine disrupting chemicals (EDCs) [3–5]. Following expert recommendations, the European Union (EU) has restricted their use during the last two decades. Three different

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regulations established between 2009 and 2014 banned all paraben use in cosmetics, except for the methyl-, ethyl-, propyl- and butyl-derivatives, and limited the allowed amounts to 0.4% and 0.8% per single or mixed usage, respectively, with even further restrictions for propyl- and butylparaben [6–8]. Moreover, in 2020 butylparaben was listed as a chemical that should be replaced with safer alternatives [9], leaving only methyl-, ethyl- and propylparaben for future applications, all of which are marketed in large quantities world-wide.

With respect to the regulation underlying the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) that was ratified into law on the July 1, 2007 [10], it became necessary to characterize the above mentioned three parabens in more detail by specific in vivo studies, for example – according to Annex VIII of REACH – in terms of the potential developmental and reproductive toxicity (DART). Since then, both methyl- and propylparaben have been very well characterized in vivo, and studies that were provided for registration by the European Chemicals Agency [11,12], as well as a recent in vivo study from 2021 [13] did not find any evidence for DART or endocrine disrupting activities. Ethylparaben, however, still needs to be characterized for its DART potential in the context of REACH, requiring that it be tested in predefined animal studies, e.g., in the extended one-generation reproduction toxicity study (EOGRTS), where large numbers of animals are required. One option that may help reduce the number of animals needed for these studies or even replace them is in vitro-based tests designed to identify developmental toxic substances [14]. To date, not a single test has been approved for regulatory purposes; however, comprehensive reviews have addressed the use of such systems in regulatory risk assessment [15,16]. Furthermore, studies describing in vitro tests that attempt to differentiate between teratogens and non-teratogens have been published [17–19], which could help in the classification of potential hazardous chemicals by adding more data on specific compounds.

In the present study, we used a test (UKN1) that recapitulates the differentiation of human stem cells to neuroepithelial precursor cells [17,20–22] to analyze the concentration-dependent effect of methyl-, ethyl-, propyl-, and butylparaben on cytotoxicity and altered genome-wide expression patterns in order to evaluate if ethylparaben shows qualitative or major quantitative differences compared to its derivates with shorter and longer side-alkyl chains. Furthermore, we compared the obtained results of ethylparaben to the data of the teratogens from our previous study [17] to assess its teratogenic potential.

2. Materials and methods

2.1. Test compounds

The compounds methyl-4-hydroxybenzoate (CAS 99-76-3), ethyl-4-hydroxybenzoate (CAS 120-47-8), propyl-4-hydroxybenzoate (CAS 94-13-3) and butyl-4-hydroxybenzoate (CAS 94-26-8), further named methyl-, ethyl-, propyl- and butylparaben, respectively, were provided by Clariant Produkte (Deutschland) GmbH, Frankfurt am Main, Germany. Valproic acid (PHR1061) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Neuroepithelial differentiation of hiPSCs and compound exposure

SBAD2 human induced pluripotent stem cells were cultured and differentiated as described previously [17] with minor changes. For compound exposure, cells were incubated for a total of 96 h with the above-described test compounds methyl-, ethyl-, propyl- and butylparaben at concentrations ranging from 0.316 to 1000 μM and valproic acid at 600 μM. On day 6, cell viability was assessed as described below before collecting the cells for RNA extraction followed by gene expression analyses. A detailed method description is given in the supplemental information.

For each compound and concentration (further named 'condition') in the UKN1 test that was analyzed with Affymetrix microarrays (see below), four independent biological replicates were generated except for the following conditions where only three biological replicates were generated: methylparaben at 31.6, 316 and 1000 μM, ethylparaben at 0.316 and 100 μM, propylparaben at 1, 3.16, 31.6 and 100 μM, butylparaben at 31.6 μM, and valproic acid at 600 μM. Furthermore, for methylparaben at 0.316 μM and propylparaben at 0.316 μM, only two replicates were generated. For each concentration of ethylparaben that was analyzed with RNA sequencing (see below), four biological replicates were generated.

2.3. Cell viability assay and cytotoxicity curves

Using the CellTiter-Blue® cell viability assay (Promega, Madison, WI, USA), cell viability and substance-induced cytotoxicity were measured. For this, cells were incubated for 20–40 min at 37 °C with a medium-mixture that was composed of 40% differentiation medium, 40% N2–S, 20% CellTiter-Blue® reagent and the growth factors SB431542, dorsomorphin and noggin. The medium-mixture was then transferred to a black 96-well tissue culture plate (Greiner bio-one, Kremsmünster, Austria) and the fluorescence intensity was measured with an Infinite M200 Pro plate reader (Tecan, Switzerland) at 594 nm in technical triplicates. The intensity of the medium-mixture incubated without cells served as background correction. Cytotoxicity curves based on the intensity measurements were calculated using the method described in Ref. [23]. Briefly, in the first normalization step, the background-corrected fluorescence intensity of each condition was normalized relative to the control sample that was set to a viability of 100%. This was done for each biological replicate separately. Then, across all biological replicates, three models were fitted to the normalized data: a monotonous four-parametric log-logistic (4pLL) model, a non-monotonous Brain-Cousens (BC) model, and a flat profile. The model with the lowest value of the Akaike information criterion (AIC) – for all data this finally was the 4pLL model – was selected and used for the second normalization step and curve fitting. The normalization of the 4pLL model was based on the left asymptote, which was set to a viability of 100%. EC_{50} values were calculated as the concentration where the fitted curve attained a viability of 50%. The R-package drc was used for the fitting of cytotoxicity curves [24].

2.4. Affymetrix microarray analysis and RNA sequencing

Total RNA that was analyzed with Affymetrix microarrays was isolated from sonicated cell lysates with the ExtractMe Total RNA-Kit (Blirt, Poland). Concentration and purity of the isolated RNA was measured with a NanoDrop2000 instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). For the microarray gene expression studies, Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were used as described previously [17,18]. Total RNA analyzed with RNA-Sequencing (RNA-Seq) was isolated with the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturers' instructions, including DNase I treatment. RNA concentrations were measured on a Qubit™ 4 Fluorometer with the RNA BR Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the RNA integrity was assessed on a 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA); all samples had an RNA integrity value (RIN) > 9.4. Strand-specific libraries were generated from 500 ng of RNA using the TruSeq Stranded mRNA Kit with unique dual indexes (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. In brief, poly (A) containing RNA molecules were isolated using magnetic beads, fragmented, and first-strand cDNA synthesis was performed using random primers, followed by second-strand cDNA synthesis, incorporating dUTP in place of dTTP to achieve strand specificity. After purification of the double-stranded cDNA fragments with AMPure XP beads (Beckman Coulter) and adenylation of the 3′ ends, unique indexing adapters were ligated to the cDNA, followed by PCR amplification. The

final libraries were quantified using the Qubit 1 × dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the library sizes were checked on an Agilent 2100 Bioanalyzer with the DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA). After library normalization, pooling, and dilution to 0.975 pM, paired-end sequencing (2 × 75 bp) was performed using the 500/550 High Output Kit v2.5 (Illumina, San Diego, CA, USA) on an Illumina NextSeq 550. The obtained FASTQ files were further processed as described below.

2.5. Data pre-processing

CEL-files of the Affymetrix microarray analysis contained raw expression values of 54,675 probe sets (PS) that were pre-processed by the frozen robust multi-array average (fRMA) algorithm, using the R-packages affy [25], frma [26], and hgu133plus2frmavecs [27] using the statistical programming software R, version 4.2.1 [28]. This algorithm consisted of the three steps background correction, normalization and summarization, resulting in pre-processed expression values for 54,675 PS for each condition and each biological replicate.

Mapping of the FASTQ files that were obtained by RNA-Seq was done using Salmon [29], version 1.4.1, with the option 'partial alignment' and the online provided decoy-aware index for the human genome hg19. To summarize the transcript reads on the gene level, the R package tximeta was used [30], resulting in data for 37,951 genes.

2.6. Differential gene expression analysis

In the differential expression analysis, the expression of paraben-treated samples was compared to control samples. In case of the Affymetrix microarray data, each treated sample was matched to a control sample of the same biological replicate to avoid batch effects. This means that for each PS the difference between the expression value of the treated and the control sample was calculated.

To analyze differential gene expression measured by Affymetrix microarrays, the R-package limma [31] was used. A moderated *t*-test, here abbreviated as "limma *t*-test", was performed where the complete set of all PS was considered for the adjustment of the variance estimates of single PS. The resulting p-values were adjusted for multiple testing by the Benjamini-Hochberg procedure [32], controlling the false discovery rate (FDR). The resulting lists for each condition contained the fold-change (FC), log2-fold change (log2-FC), and the unadjusted and FDR-adjusted p-values of the limma *t*-test for all 54,675 PS. PS with a FC larger than 2 or smaller than 0.5 and FDR-adjusted p-value smaller than 0.05 were considered to be significant.

Differential expression analysis of the pre-processed RNA-Seq data was done using the R package DESeq2 [33]. A general linear model was fitted to the data. Using default settings, the independent filtering step which preceded the Benjamini-Hochberg procedure removed low-expressed genes to increase the power of the FDR-adjustment of the p-values. Resulting log2-FCs of the differential expression analyses were shrunken towards zero with the adaptive shrinkage approach [34] to remove expression changes that were mostly influenced by random noise, and FC were obtained by calculating 2 to the power of the shrunken log2-FC. The resulting list for each condition summarized the shrunken FC and log2-FC, and the unadjusted and FDR-adjusted p-values for 37,951 genes with a given Ensembl-ID. Genes with a FC larger than 2 or smaller than 0.5 and FDR-adjusted p-value smaller than 0.05 were considered to be differentially expressed genes (DEGs).

2.7. Translation of PS and ensembl-ID-genes into unique HGNC-genes

The Affymetrix microarray analyses were conducted on a PS-level, while the RNA-Seq analyses were conducted on an Ensembl-ID-based gene-level. To be able to compare the expression results of both methods, the PS and Ensembl-ID-genes were translated to gene names based on the HUGO gene nomenclature committee (HGNC) [35]. The 54,675 PS were translated to 43,135 genes, among which 20,862 unique gene names could be found, and the 37,951 Ensembl-ID-genes were each translated to their respective HGNC names, which yielded 35,940 unique HGNC-genes. In the next step, the lists that contained the genes for each condition were prepared such that each HGNC-gene appeared only once in each list: if an HGNC-gene had more than one entry in a gene list and at least one entry had an adjusted p-value smaller than 0.05 ('significant'), all entries with an adjusted p-value larger than 0.05 ('non-significant') were removed; among all significant entries, only the entry with the highest absolute log2-FC was retained while the others were removed; if only non-significant entries for an HGNC-gene were found in a list, only the one with the highest absolute log2-FC was retained while the others were removed.

2.8. Venn diagrams, GO groups and top genes

In order to compare the gene expression results of Affymetrix microarrays and RNA-Seq, Venn diagrams were created, and for each element of the Venn diagram, overrepresented Gene Ontology (GO) groups and top genes were identified. This was done for RNA-seq HGNC-genes that were significant for ethylparaben at 1000 µM only ('ethylparaben only'), for the translated HGNC-genes that were significant across the group of teratogens at the highest non-cytotoxic concentrations from [17] only ('teratogens only'), and for the overlap of both ('shared genes'). This was repeated three times, where either all genes, only upregulated, or only downregulated genes were considered.

Overlap ratios of Venn diagram elements were calculated as previously introduced [36]: with n_{universe} denoting the intersection of all genes that could be maximally measured by gene arrays and RNA-Seq (i.e., 17,448 genes); n_1 and n_2 denoting the number of DEGs found in test condition 1 and 2, respectively, excluding the genes measured exclusively in gene arrays or RNA-Seq; and O denoting the size of the overlap. The overlap ratio was calculated as $(O \cdot n_{\text{universe}})/(n_1 \cdot n_2)$. A value of 1 indicates a random overlap, and values larger than 1 indicate the factor by which the overlap is larger than randomly expected.

GO group overrepresentation analysis were conducted using the R package topGO [37], and genes were assigned to GO groups according to the ontology 'Biological Process'. Using Fisher's exact test, it was tested whether more genes in the respective GO group were DEGs than expected at random. A bottom-up ('elim') approach was used, where genes that were already contained in a more specific GO group according to the GO hierarchy were not considered again in more general groups [38]. Significant GO groups for each gene set were determined, where a group was considered to be significant if the FDR-adjusted p-value of Fisher's test was smaller than 0.05.

Top genes were identified by sorting the DEGs according to their (absolute) log2-FC in a decreasing way when considering all genes or upregulated genes only, and in an ascending way when considering downregulated genes. For 'ethylparaben Only', only a sorting according to the log2-FC was conducted. For 'shared genes' and 'teratogens only', the first level of sorting was obtained by calculating for each DEG the number of teratogens that significantly deregulated the gene. The second level of sorting was obtained by calculating the mean (absolute) log2-FC across all compounds for each DEG.

2.9. RT-qPCR analysis

For the measurement of gene expression changes in UKN1 with RT-qPCR, RNA was first transcribed into complementary deoxyribonucleic acid (cDNA) with the "High-Capacity cDNA Reverse Transcription Kit" (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions. RT-qPCR measurements were performed using QuantiFast SYBR® Green PCR master mix (Qiagen, Germany) and self-designed primers for the genes *TFAP2B* and *TBP* that were obtained from Eurofins, Luxembourg. Fold-changes were calculated by using the $2^{-\Delta\Delta CT}$-method [39]. Primer sequences (5'→ 3') were as follows:

TFAP2B forward: GGGGAGATCTTTGCGAGAAAGG; reverse: CTGTGTGCTGCCGGTTCAAATA; *TBP* forward: GGGCACCACTC-CACTGTATC; reverse: GCAGCAAACCGCTTGGGATTATATTCG.

3. Results

3.1. Study design

The previously-published UKN1 test [17] was used to assess the effect of paraben-exposure on neuroepithelial precursor cells (NEPs) that were differentiated from human induced pluripotent stem cells (hiPSCs). A schematic overview of the UKN1 test is given in Fig. 1. The cells were exposed to methyl-, ethyl-, propyl- and butylparaben for four days during the neuroepithelial differentiation process. The concentrations used were between 0.316 and 1000 µM, which were based on peak plasma concentrations measured in humans exposed to parabens (Table 1). After an incubation period of 4 days and a 2-day washout phase, paraben-induced effects on the cells were examined by measuring cytotoxicity and genome-wide expression changes.

3.2. Cytotoxicity of high concentrations of parabens

The CellTiter-Blue® cell viability assay was used to analyze paraben-induced cytotoxicity on NEPs. Cytotoxicity of NEPs occurred at concentrations that were much higher than those reported in human plasma (Fig. 2, Table 2, Supp. excel file 'Cytotoxicity'). For instance, the lowest concentration of methylparaben where cytotoxicity was observed in NEPs was approximately 100 µM, which was 20-fold higher than the highest reported human plasma concentration of 5.74 µM [42,43]. For butylparaben, the onset of cytotoxicity at approximately 10 µM was about 5500-fold higher than the reported plasma concentration of 0.0018 µM [42,44]. The cytotoxicity of parabens increased with the increasing length of the side-alkyl-chain. Accordingly, methylparaben with an $–CH_3$-side-chain had the highest EC_{50}-value of 906 µM – and therefore the lowest cytotoxic effect – whereas butylparaben with an $–CH_2CH_2CH_2CH_3$-side chain led to a 15-fold higher cytotoxicity and an EC_{50}-value of 63 µM. The EC_{50}-value of cytotoxicity for ethylparaben was 697.5 µM. In a second experiment performed about three years later (for RNA-seq analysis; see below) cytotoxicity was even slightly lower, so that 1000 µM ethylparaben in the RNA-Seq experiments reduced the viability by only 40% compared to 100% in the microarray experiments.

Table 1
Plasma and blood concentrations of parabens in neonates and umbilical cord blood from published biomonitoring studies.

Substance	Plasma and blood concentrations [µg/l]	Blood and plasma levels [µM]	Reference
Methylparaben	a) 0.89–210 (mean 54.8) b) Median: 12–15, maximum: 311-874	0.006–5.74	a) [42] b) [43]
Ethylparaben	a) 0.15–3.82 (mean 0.7) b) 0.01–9.95 (mean 0.68)	0.00006–0.06	a) [42] b) [44]
Propylparaben	a) 0.27–31.8 (mean 4.28) b) 0.03–78.12 (mean 5.59) c) Median: n/d, maximum 134-147	0.00017–0.82	a) [42] b) [44] c) [43]
Butylparaben	a) 0.09–0.26 (mean 0.09) b) 0.01–0.35 (mean 0.07)	0.00005–0.0018	a) [42] b) [44]

Therefore, ethylparaben appears to be less cytotoxic than propyl- and butylparaben, and similarly or slightly more cytotoxic than methylparaben.

3.3. Effect of parabens on gene expression

The influence of paraben exposure on gene expression in NEPs was analyzed using Affymetrix microarrays where the expression of 54,675 probe sets for each paraben and paraben concentration (further named 'condition') were measured. Valproic acid (VPA), which is known to induce strong gene expression changes in NEPs at non-cytotoxic concentrations, such as 600 µM [21,22], served as a positive control. Transcriptome-wide gene expression changes were illustrated using volcano plots for VPA and ethylparaben at 100 µM (Fig. 3). Plots for all conditions can be found in the supplemental information. Statistical analyses identified all probe sets for each condition that were significantly deregulated with an absolute fold-change (FC) > 2 and a false discovery rate (FDR) adjusted p-value < 0.05. These are indicated as red dots in the volcano plots. Strikingly, the majority of conditions, except for 0.316 µM methylparaben and 100 µM ethylparaben, did not induce any significant gene expression changes and were not cytotoxic (Table 2). In contrast, the positive control substance VPA had a considerable effect on gene expression and induced significant expression changes in 599 probe sets.

We went on to investigate if the observed significant changes in the

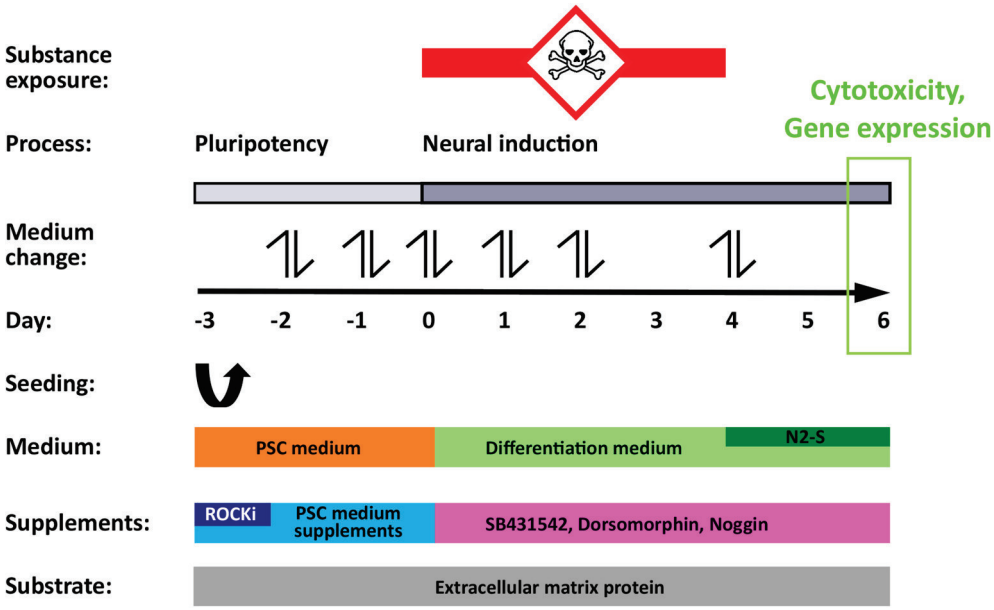

Fig. 1. Schematic overview of the UKN1 developmental toxicity test. Pluripotent stem cells were cultured for three days after seeding and then differentiated to neuroepithelial precursor cells in six days. During the differentiation process, cells were incubated with parabens for 4 days followed by a washout period of 2 days. On day 6 of the protocol, the viability of the cells was determined and cells were harvested for gene expression analysis. The indicated culture medium supplements were added daily and medium changes were performed at the days −2, −1, 0, 1, 2 and 4. PSC medium = Pluripotent stem cell medium; ROCKi = Rho-kinase inhibiting growth factor. Modified from [40,41].

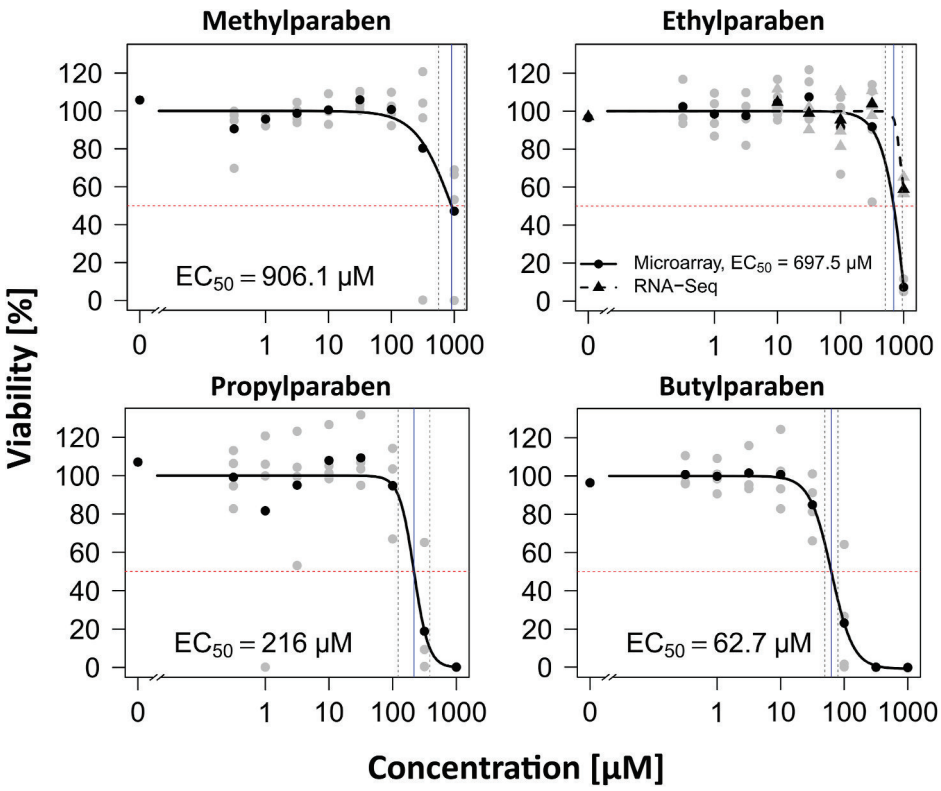

Fig. 2. Cytotoxicity of parabens in the UKN1 assay system. Neural progenitor cell viability is indicated in relation to paraben concentrations. Gray data points represent viability data obtained from one biological replicate and black data points represent mean values of these replicates. The continuous vertical blue lines mark the EC_{50} values on the x-axis, and the dashed vertical blue lines indicate the confidence intervals. The dashed horizontal lines mark the 50% viability level. For ethylparaben, two curves are plotted since a second experimental series was performed (triangles). This second set of experiments resulted in a slightly lower cytotoxicity than the first experiment. An EC_{50} was not calculated for the second experiment since 50% cytotoxicity was not reached. The samples from the first batch (continuous curve, dots) were used for gene expression analysis with microarrays (as all replicates of methyl-, propyl- and butylparaben); whereas, RNA from the second batch (dashed curve, triangles) was measured via RNA-sequencing. Raw data are summarized in the supplementary excel file 'Cytotoxicity'.

Table 2
Number of significantly deregulated probe sets (=SPS) and corresponding genes (=DEGs, based on unique gene names) in a gene array analysis of NEPs incubated with valproic acid and parabens.

Concentration [µM]	Number of significantly up-/downregulated probe sets and genes									
	VPA		Methylparaben		Ethylparaben		Propylparaben		Butylparaben	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
600	SPS: 298 DEGs: 216	SPS: 301 DEGs: 209	–	–	–	–	–	–	–	–
0.316	–	–	SPS: 5 DEGs: 2	SPS: 1 DEGs: 1	0	0	0	0	0	0
1	–	–	0	0	0	0	0	0	0	0
3.16	–	–	0	0	0	0	0	0	0	0
10	–	–	0	0	0	0	0	0	0	0
31.6	–	–	0	0	0	0	0	0	0	0
100	–	–	0	0	SPS: 80 DEGs: 55	SPS: 115 DEGs: 85	0	0	cytotoxic	
316	–	–	0	0	0	0	cytotoxic		cytotoxic	
1000	–	–	0	0	cytotoxic		cytotoxic		cytotoxic	

195 significant probe sets caused by 100 µM ethylparaben were reproducible or represented a unique experimental effect, especially as the higher (and still non-cytotoxic) concentration of 316 µM did not cause significant gene expression changes. Therefore, experiments were repeated with a range of ethylparaben concentrations and analyzed using genome-wide RNA sequencing (RNA-Seq) to validate the results obtained in the microarray studies. Volcano plots of RNA-Seq measurements can be found in the supplemental information. The RNA-Seq revealed that only 1000 µM ethylparaben (a cytotoxic concentration) induced a high number of significantly (FC $>$ 2, p-value $<$ 0.05) deregulated genes (Fig. 4, Table 3). These data were further confirmed using RT-qPCR to measure expression of *TFAP2B,* a transcription factor of the AP-2 family, which was only upregulated by VPA and high paraben concentrations (Supp. Fig. 1, Supp. excel file 'RT-qPCR'). Taken together, the obtained results do not indicate that the individual parabens have different effects on the gene expression of NEPs, since no expression changes were observed at non-cytotoxic concentrations. The six significant probe sets induced by methylparaben at 0.316 µM (Table 2) were not included in any further analysis.

3.4. Genes and gene networks affected by a high concentration of ethylparaben

Next, we addressed to what extent the gene expression alterations obtained by RNA-Seq for ethylparaben at 1000 µM, i.e. under cytotoxic conditions, were similar to those induced by teratogenic substances found in our previous study, where we tested a total of 39 teratogenic and non-teratogenic compounds [17]. For this purpose, we used data from 12 teratogenic substances at the highest tested, non-cytotoxic concentration, i.e., 9-cis retinoic acid at 20 µM, acitretin at 24 µM,

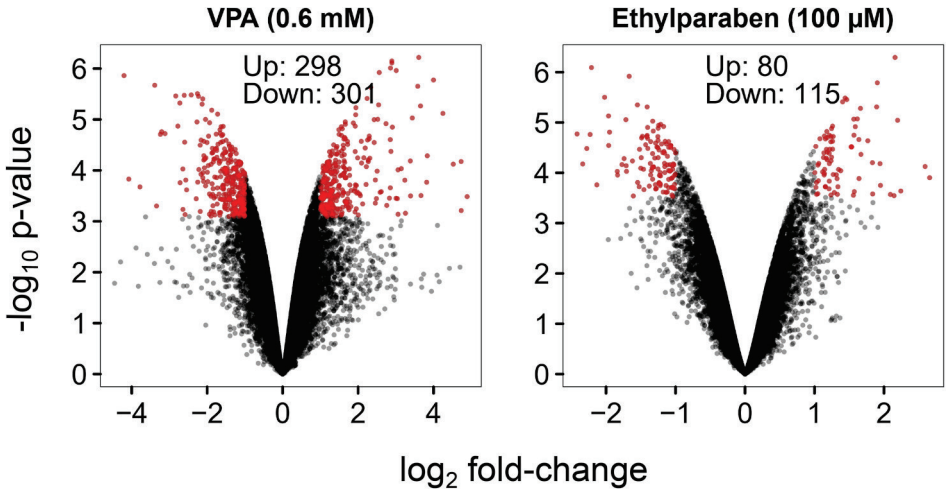

Fig. 3. Volcano plots of deregulated probe sets in UKN1 cells incubated with 600 µM VPA and 100 µM ethylparaben obtained with Affymetrix gene array measurements. Each dot (54,675 in total) represents a probe set. The x-axis gives the log2-values of the fold-change of the deregulated probe sets (compared to control cells), and the y-axis the negative log10-value of the corresponding p-values based on the limma analysis. Significantly deregulated probe sets (with an FDR-adjusted p-value <0.05 and an absolute log2-fold-change>1) are highlighted in red, and the total numbers of significantly up- and downregulated probe sets are indicated.

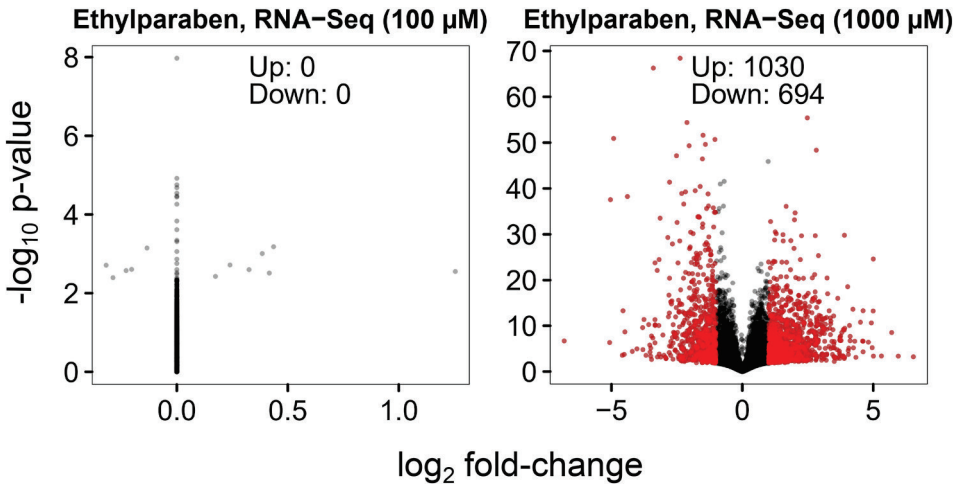

Fig. 4. Volcano plots of deregulated genes in cells incubated with 100 µM and 1000 µM ethylparaben obtained with Illumina RNA-Sequencing. Each dot represents a gene. In total, Illumina RNA-seq measured the gene expression of 37,951 different genes (based on their Ensembl ID). The x-axis gives the shrunken log2-values of the fold-change of the deregulated genes (compared to control cells), the y-axis provides the negative log10-value of the corresponding p-values based on the analysis of differentially expressed genes. Significantly deregulated genes (with an FDR-adjusted p-value <0.05 and an absolute log2-fold-change>1) are highlighted in red, and the total numbers of significantly up- and downregulated genes are indicated.

Table 3
Numbers of significantly deregulated genes, either based on Ensembl IDs or unique gene names (=DEGs), in RNA-seq measurements of NEPs incubated with ethylparaben.

Concentration [µM]	Upregulated genes	Downregulated genes
0.316	0	0
1	0	0
3.16	0	0
10	0	0
31.6	0	0
100	0	0
316	0	1
1000	IDs: 1030 DEGs: 1027	IDs: 694 DEGs: 693

carbamazepine at 190 µM, entinostat at 4 µM, favipiravir at 7.6 mM, isotretinoin at 34 µM, leflunomide at 370 µM, methotrexate at 20 µM, retinol at 20 µM, teriflunomide at 370 µM, trichostatin A at 0.2 µM and valproic acid at 1000 µM. The comparison was performed for differentially expressed genes (DEGs) and Gene Ontology (GO) groups. To enable this comparison, we first translated the results of the Affymetrix microarrays and Illumina RNA-Sequencing from the levels of probe sets and Ensembl IDs, respectively, to standardized gene names. Furthermore, we also considered that the microarrays and RNA-Seq measurements covered different numbers of genes, and that only 44% gene overlap was observed with both (Supp. Fig. 2). When we compared the expression of all deregulated genes for ethylparaben and all genes deregulated by the 12 teratogens (Supp. excel file 'DEGs and top genes'), we found that 1018 genes (59%) induced by ethylparaben (1000 µM) overlapped with the genes induced by all 12 teratogens (Fig. 5A). Of the remaining 702 genes (41%) that did not overlap, 161 genes (9%) were exclusively measured by RNA-Seq. The teratogens additionally induced 3705 different genes, of which 305 were exclusively measured by microarrays. Based on these findings, we investigated which GO groups were overrepresented by the DEGs only deregulated by ethylparaben, by the teratogens and by the shared DEGs, respectively (Supp. excel file 'GO groups'). Impressively, 96% of all significantly overrepresented GO groups were found for the subset of 'shared genes' of ethylparaben and the 12 teratogens (Fig. 5B). Several of the GO groups among those with the lowest p-values were related to developmental processes of the ectodermal and mesodermal lineages, such as 'neural crest cell migration', 'innervation', 'angiogenesis', 'nephron tubule formation' and 'aortic valve morphogenesis'. With respect to the subset of genes influenced by teratogens alone (and not by ethylparaben), only five GO groups (4%) were found, which were also related to neuroectodermal development, such as 'neuron migration', 'neural precursor cell proliferation' and 'forebrain neuron development'. The GO group 'axon guidance' was overrepresented in both subsets ('shared genes' and 'teratogens only'). Interestingly, no significant GO groups were found for genes only influenced by ethylparaben (and not by the teratogens).

Among the top shared genes deregulated by at least 10 teratogenic substances and ethylparaben are the secreted autocrine factors SEMA3C

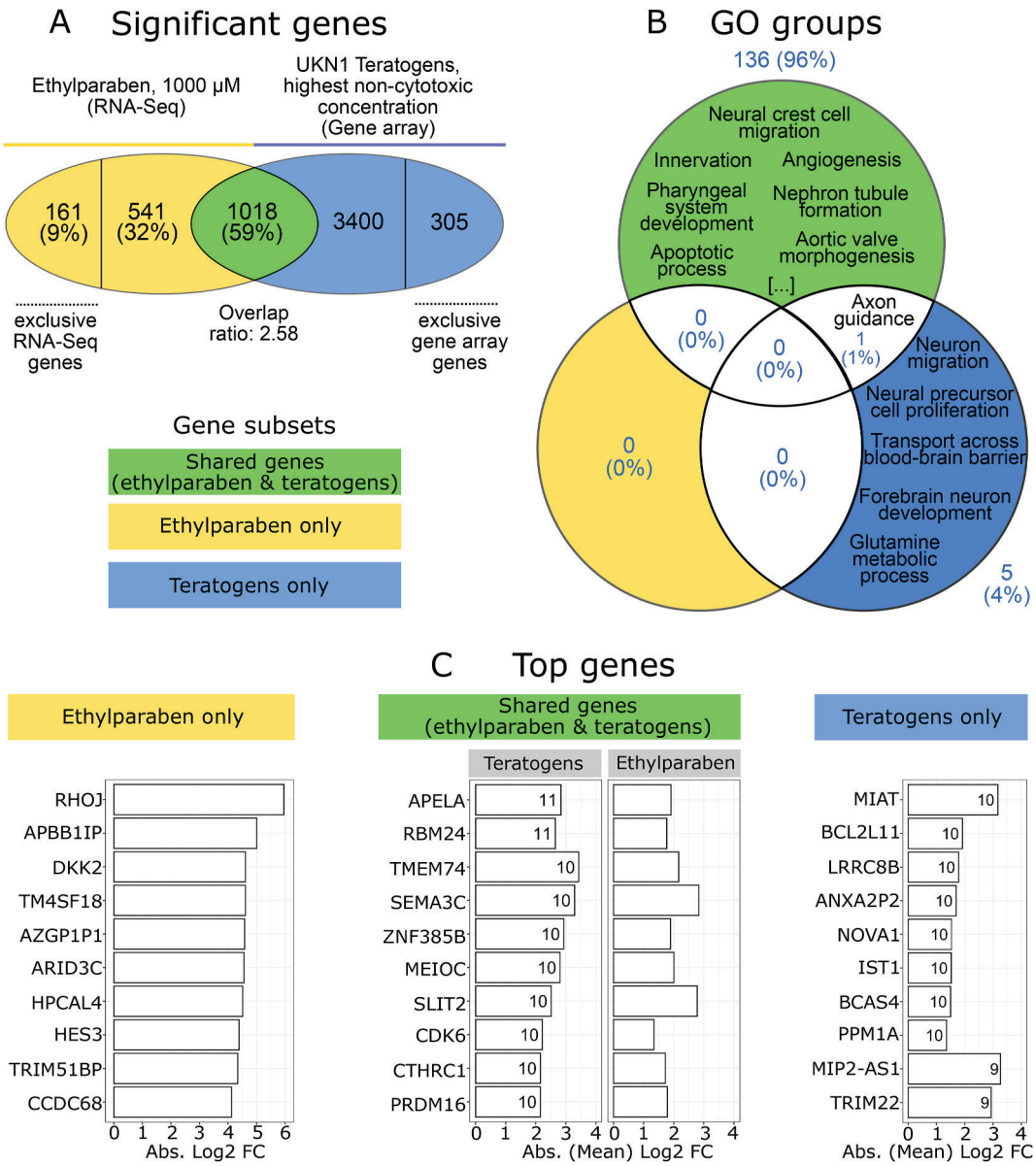

Fig. 5. Comparison of deregulated genes and overrepresented biological networks caused by ethylparaben at 1000 µM and teratogens in the UKN1 system. (A) Number of significantly deregulated genes (based on unique gene names) in NEPs after incubation with ethylparaben at 1000 µM, as well as all genes significantly deregulated upon exposure to 12 teratogens. For the teratogens, data were obtained from [17], and the sum of all deregulated genes across all teratogens at their highest non-cytotoxic concentrations was calculated. Three gene sets were defined: 'Shared genes' – 1018 genes from the overlap of ethylparaben and teratogens (green); 'ethylparaben only' – 702 genes uniquely deregulated upon exposure to 1000 µM ethylparaben (yellow); 'teratogens only' – 3705 genes deregulated only by the teratogens (blue). The fraction of genes that were detected by RNA-Seq only in the ethylparaben gene set or by gene arrays only in the teratogen gene set is indicated. For the calculation of the overlap ratio, only genes that were measurable by both gene array and RNA-seq, were considered. **(B)** Overrepresented GO groups in the three gene sets. Number of significant GO groups are given in blue. Names of GO groups were shortened. Lists with all GO groups and full names are given in the supplementary excel file 'GO groups'. **(C)** Top genes of the three gene sets. For 'ethylparaben only', genes were sorted by the absolute value of the log2 fold-change. For 'shared genes' and 'teratogens only', genes were first sorted by the number of deregulating teratogens (numbers are given in the bars), and then by the absolute value of the log2 fold-change. For the teratogens, the mean of the absolute log2 fold-change was calculated across the deregulating teratogens. Lists of all DEGs are given in the supplementary excel-file 'DEGs and top genes'. NEP = Neuroepithelial precursor cells.

that plays a role in neurogenesis, and the slit family member SLIT2, a secreted glycoprotein that is relevant during neural development (Fig. 5C, Supp. excel file 'DEGs and top genes'). For these two genes, ethylparaben caused a fold-change that was comparable to the mean of the teratogenic substances (log2 FC ≈ 3). Among the top genes that were only deregulated by ethylparaben are the proteins DKK2 and ARID3C, which are involved in embryonic development and patterning, as well as the transcription factor HES3 that regulates neural development.

In addition to the genes and GO groups listed above that were obtained by analyzing all genes (up- and downregulated genes), the same analysis was repeated for only the up- or only the downregulated genes (Supp. Figs. 3 and 4, respectively, Supp. excel file 'DEGs and top genes'). Here, more shared genes were found to be common among the upregulated than the downregulated genes (694 (67%) vs. 288 (42%)), and the overlap ratio was also higher (4.92 vs. 3.57) (Supp. Figs. 3A and 4A, respectively). Remarkably, shared GO groups were only found for the upregulated genes (Supp. Fig. 3B), except for 'axon guidance' for the downregulated genes (Supp. Fig. 4B). Furthermore, the number of significant GO groups was as high as for the GO group enrichment analysis that considered all genes (141 and 137, respectively). In

conclusion, the high overlap between ethylparaben and the teratogens as well as the high number of GO groups associated to developmental pathways point to similar effects of the substances on the gene expression of NEPs. It is, however, important to consider that this occurred only at the cytotoxic concentration of 1000 μM that is orders of magnitudes above the concentration to which humans are exposed.

4. Discussion

Parabens have been widely used in industrial products since the 1950s, primarily as preservatives in food and cosmetic products [1]. However, their safety is currently discussed as several studies have suggested that they pose a potential risk for human health, especially as endocrine-disrupting chemicals [3,4]. Unfortunately, in order to conduct risk assessment of parabens to evaluate their safety, extensive in vivo studies are required, which are cost-intensive and call for large numbers of experimental animals. Therefore, in the present work, we utilized the recently-published UKN1 test, a stem-cell based in vitro assay that allowed the classification of developmental toxic substances at in vivo relevant concentrations with an accuracy of 90% [17].

Using cytotoxicity as a readout, the EC_{50} of methyl-, ethyl-, propyl- and butylparaben were 906 μM, 698 μM, 216 μM and 63 μM, respectively, illustrating that cytotoxicity increased with increasing length of the alkyl side chain. The same parabens did not significantly alter gene expression in the microarray studies, except for ethylparaben at 100 μM and methylparaben at 0.316 μM, whereas the latter one only induced 3 DEGs. Nevertheless, a second series of experiments and subsequent RNA-Seq analysis of ethylparaben showed that massive gene expression changes occurred only at cytotoxic concentrations and that the previous results of 100 μM ethylparaben were unique and not reproducible. In this second series, ethylparaben was cytotoxic at 1000 μM, reducing vitality by about 40%. This concentration also caused up- and down-regulation of 1027 and 693 genes, respectively, after false discovery rate (FDR) adjustment. The next lower tested concentration of 316 μM caused no cytotoxicity, and significantly altered the expression of only one gene. At all tested lower concentrations (100 μM; 31.6 μM; 10 μM; 3.16 μM; 1.0 μM and 0.316 μM), neither cytotoxicity nor FDR-adjusted significant gene expression changes were observed for ethylparaben. Thus, the parabens differ from the here-applied positive control substance VPA, since the latter clearly caused gene expression alterations below cytotoxic thresholds [21,22].

An important step in risk assessment is the comparison of the concentration of a compound that causes a positive test result in an in vitro test (for example, cytotoxicity or gene expression changes) to the concentrations of this compound that may occur in the blood. Biomonitoring studies investigating umbilical cord blood plasma samples from multiethnic cohorts reported 60 nM as the highest detected concentration of ethylparaben [42]. Comparing this concentration to the highest tested concentration that caused no cytotoxicity nor gene expression alterations detected by RNA-Seq, namely 100 μM, resulted in a 'margin of exposure' factor of 1670, which is extremely high. Furthermore, it should be considered that the point-of-departure of 100 μM is rather conservative, since only a single gene was significantly deregulated at the next higher tested concentration of 316 μM.

In our previous work, in which the UKN1 assay was used to analyze a set of 39 teratogenic and non-teratogenic compounds, we identified a large number of significant gene expression alterations upon teratogen-exposure [17]. By using the microarray data of the 12 teratogenic substances that had the strongest influence on gene expression, we found a total of 4723 significantly deregulated genes. When we compared these genes to the 1720 genes detected by RNA-Seq to be deregulated by 1000 μM ethylparaben, an overlap of 1018 genes corresponding to 59% was obtained, which was 2.58-fold higher than randomly expected. This result shows that investigating ethylparaben in the UKN1 assay leads to gene expression changes that are also altered by teratogens. It should, however, be considered that this effect only occurs at cytotoxic concentrations of ethylparaben, which are 1670-fold above the highest concentrations reported in currently-available biomonitoring studies [42].

In conclusion, analyses of the four parabens reveal that cytotoxicity is related to the length of the alkyl side chain and gene expression changes occur only at cytotoxic or close to cytotoxic concentrations. Since methyl- and propylparaben tested negatively in comprehensive DART animal experiments, the present in vitro study provides no evidence that the ethyl derivate may lead to qualitatively different effects, indicating a high likelihood of negative in vivo test results.

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Author contribution

Project conceptualization and experimental design was provided by Florian Seidel, Jan G. Hengstler, Susann Fayyaz and Reinhard Kreiling. Florian Seidel performed the cell culture, hiPSC differentiation and RNA extraction with the help of Andreas Scholtz-Illigens. Microarray studies were conducted by Anna Cherianidou. RNA sequencing was performed by Katharina Derksen with the help of Andreas Scholtz-Illigens. Microarray and RNA-Seq data were analyzed by Franziska Kappenberg with the help of Karolina Edlund. Marcel Leist, Agapios Sachinidis, Jörg Rahnenführer, Karolina Edlund and Patrick Nell provided substantial advice on the project conceptualization, experimental design and data analysis. Rosemarie Marchan critically reviewed the manuscript. Florian Seidel and Jan G. Hengstler wrote the paper.

Declaration of competing interest

The parabens tested in this study were provided by Clariant Produkte (Deutschland) GmbH. Companies of the Clariant Group produce and market parabens, among other products. Susann Fayyaz and Reinhard Kreiling are employees of Clariant Produkte (Deutschland) GmbH.

Data availability

Research data are either available in the supplemental files or in gene expression databases. The raw data generated with Affymetrix gene arrays during the current study are accessible in the Gene Expression Omnibus (GEO) database under GSE233332. Illumina RNA-sequencing raw data will be available at the NCBI BioProject database under the title "RNA-seq of human induced pluripotent stem cell (iPSC)-derived neuroectodermal cells exposed to ethylparaben., Apr 19 '23" with the accession number PRJNA956726 by the 31st December, 2024 (https://www.ncbi.nlm.nih.gov/sra/PRJNA956726).

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Supplemental information

SOP UKN1 protocol; Figs. S1–S4; Volcano plots; Excel-files 'Cytotoxicity', 'GO groups', 'DEGs and top genes' and 'RT-qPCR'.

Abbreviations

DEG – differentially expressed gene
GO – Gene ontology
NEP – neuroepithelial precursor cell
PS – Probe set
VPA – valproic acid

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