Transcriptomic Concentration-Response Evaluation of Valproic Acid, Cyproconazole, and Hexaconazole in the Neural Embryonic Stem Cell Test (ESTn)

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Alternative developmental toxicity assays are urgently needed to reduce animal use in regulatory developmental toxicology. We previously designed an in vitro murine neural embryonic stem cell test (ESTn) as a model for neurodevelopmental toxicity testing (Theunissen et al., 2010). Toxicogenomic approaches have been suggested for incorporation into the ESTn to further increase predictivity and to provide mechanistic insights. Therefore, in this study, using a transcriptomic approach, we investigated the concentration-dependent effects of three known (neuro) developmental toxicants, two triazoles, cyproconazole (CYP) and hexaconazole (HEX), and the anticonvulsant valproic acid (VPA). Compound effects on gene expression during neural differentiation and corresponding regulated gene ontology (GO) terms were identified after 24 h of exposure in relation to morphological changes on day 11 of culture. Concentration-dependent responses on individual gene expression and on biological processes were determined for each compound, providing information on mechanism and concentration-response characteristics. All compounds caused enrichment of the embryonic development process. CYP and VPA but not HEX significantly enriched the neuron development process. Furthermore, specific responses for triazole compounds and VPA were observed within the GO-term sterol metabolic process. The incorporation of transcriptomics in the ESTn was shown to enable detection of effects, which precede morphological changes and provide a more sensitive measure of concentration-dependent effects as compared with classical morphological assessments. Furthermore, mechanistic insight can be instrumental in the extrapolation of effects in the ESTn to human hazard assessment.

Key Words: embryonic stem cells; neural embryonic stem cell test (ESTn); toxicogenomics; alternative test method; neural differentiation.

Due to the high use of experimental animals needed for regulatory developmental toxicity testing (Barrow, 2009; van der Jagt et al., 2004), there is increased momentum to develop, refine, and establish in vitro screening assays to replace classical in vivo tests (Scholz et al., 1999). In the embryonic stem cell test (EST), murine embryonic stem cells (mESC) are exposed to compounds during cardiomyocyte differentiation, giving an indication of developmental toxicity by scoring foci of beating cells (Scholz et al., 1999). The EST is widely being evaluated as a developmental toxicity screening assay in both chemical and pharmaceutical industry. The European Centre for Validation of Alternative Methods (ECVAM) validation study found this test to provide a predictivity score of 78%, but subsequent validation studies were less successful (Marx-Stoelting et al., 2009; Paquette et al., 2008). To improve the predictivity of the EST, it was advised to add additional differentiation lineages to the testing strategy, such as neural and osteoblast differentiation, and to use objective and mechanistic informative molecular techniques to determine compound effects (Marx-Stoelting et al., 2009; Spielmann et al., 2006). In this line of thought, we developed an 11-day mESC neural differentiation protocol (ESTn). Using this assay, we were able to detect specific adverse effects of the neurodevelopmental toxicant methylmercury (MeHg) (Theunissen et al., 2010), a compound that was misclassified in the ECVAM EST validation study (Genschow et al., 2004). Further investigations using additional developmental toxicants are needed to establish the applicability domain of this test for developmental toxicity testing.

Toxicogenomic approaches might be incorporated into alternative test systems to further increase predictivity and provide mechanistic insight. In our initial transcriptomic study in the ESTn, we characterized gene expression changes over time in nonexposed cultures and described the specific time-dependent
gene expression profile after MeHg exposure (Theunissen et al., 2011). Recent transcriptomic studies in the cardiac EST (ESTc) have shown that testing a compound at one concentration provides only limited information on the effect profile of the compound (van Dartel et al., 2010a, 2011b). A recent study describing the effects of flusilazole in the ESTc illustrated the benefit of concentration-response testing in EST transcriptomics experiments (van Dartel et al., 2011a).

Therefore, in this study, a similar concentration-response approach was performed to discern effects of three model compounds on neural differentiation in the ESTn: the triazole fungicides cyproconazole (CYP) and hexaconazole (HEX) and the anticonvulsant valproic acid (VPA). CYP and HEX have been identified to be developmentally toxic in vivo (de Jong et al., 2011; Machera, 1995). In rats, CYP increased the prevalence of skeletal malformations, including delayed ossification of skull bones, cleft palate, hydrocephalus, and hydropsphrosis (Machera, 1995; de Jong et al., 2011). In the cardiac differentiation EST, CYP has been shown to induce neural differentiation, inducing deviation from the normal differentiating pattern. In rats, HEX caused skeletal malformations, however, no specific effects on neural development have been observed in vivo or in vitro (de Jong et al., 2011). The widely used anticonvulsant and histone deacetylase (HDAC) inhibitor VPA is well known for its induction of central nervous system (CNS) congenital abnormalities, including spina bifida and other neural tube defects (NTDs) but also heart anomalies and skeletal malformations (Duncan, 2007; Meador et al., 2008).

Using these three model compounds (CYP, HEX, and VPA), we investigated the concentration-dependent effects of compound exposure in the ESTn on gene expression in relation to neural differentiation in vitro and to morphological effects observed in vivo. Using a toxicogenomic approach, we are able to discriminate between compound classes and between mechanisms of action on the basis of differential gene expression during neural differentiation.

**MATERIALS AND METHODS**

**Embryonic stem cell culture and neural differentiation.** Murine embryonic stem cells (mESC) (ES-D3; ATCC, Rockville, MD) were routinely subcultured every 2–3 days and grown as a monolayer in Dulbecco’s Modified Eagle Medium-based (Gibco BRL, Gaithersburg, MD) medium supplemented with leukemia inhibiting factor (Chemicon, Temecula, CA) at a final concentration of 1000 units/ml. The cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. Induction of neural differentiation was performed as described earlier (Theunissen et al., 2011).

**Resazurin cell viability assay.** To determine compound effects on cell viability, a resazurin dye reduction assay (Promega, Leiden, The Netherlands) was used as a measure for the number of viable cells, as described previously (Theunissen et al., 2010). Cyproconazole (CYP) (CAS number 93651-06-5; Sigma-Aldrich, Zwijndrecht, The Netherlands), hexaconazole (HEX) (CAS number 79983-71-4; Sigma-Aldrich), and valproic acid sodium salt (VPA) (CAS number 1069-66-5; Sigma-Aldrich) were diluted in dimethyl sulfoxide (DMSO), and cell cultures (including controls) were exposed to a final DMSO concentration of 0.1%, which did not affect cell viability. Viability was tested in the following concentration ranges: CYP 0.1–300 μM, HEX 0.01–300 μM, and VPA 0.1–2.5 mM. Tests were performed in triplicate.

**Treatment and morphological scoring for effects on neurite outgrowth.** Highest concentrations tested were selected on basis of the resazurin viability assay, wherein the highest concentration provided a minimum viability of 80%, or 1000 μM, chosen as the highest concentration feasible in vivo. A concentration of 100 μM CYP and 25 μM HEX reduced viability to 80% and 1000 μM VPA did not affect cell viability in the ESTn. For both morphological and gene expression assessments, compounds were tested at the following concentrations: CYP 1.5, 6.0, 25, and 100 μM; HEX 0.5, 1.5, 6.0, and 25 μM; VPA 15, 60, 250, and 1000 μM. Control embryoid bodies (EB) were treated with 0.1% DMSO. For morphological scoring, cultures were treated for 72 h from initiating suspension culture on day 3 of the protocol until the start of the serum-free period on day 6 (Theunissen et al., 2011). Morphological effects were determined by assessment of the extent of neurite outgrowth from the EB, at the end of the differentiation protocol (day 11), observed using an IX51 inverted microscope (Olympus, Zoeterwoude, The Netherlands) with CellD software (Olympus). Morphology of neurite outgrowth was scored as the percentage of neural corona surrounding each EB, irrespective of the distance of outgrowth from the EB. For scoring neurite outgrowth, a cutoff was chosen at or ≥75% neurite outgrowth around the EB, based on historical control data. For each concentration, three cultures each with 30–40 EB were assessed. Parallel cultures were harvested for gene expression analysis at earlier time points.

**RNA isolation and whole genome expression profiling.** Exposed differentiation cultures were treated with concentrations as described earlier from day 3 of the protocol onward and sampled after 24-h exposure (day 4) (eight replicates per group). The 24-h exposure was chosen as the time point expressing the least variability between samples as observed in our earlier time-course study with the neurodevelopmental toxicant MeHg (Theunissen et al., 2011). RNA from control differentiation cultures was sampled on protocol days 3 (eight replicates), 4 (five replicates), and 5 (five replicates). Gene expression background over time correlated well with previous studies (Theunissen et al., 2011). Cells (~30 to 40 EB/sample) were directly collected in RNA Protect (Qiagen Benelux, Venlo, The Netherlands) to stabilize RNA, and total RNA was purified using the Qiagen RNeasy Plus Mini Kit (Qiagen Benelux) following manufacturer’s instructions. RNA quantity was determined using the NanoDrop Spectrophotometer (Isogen Life Science, De Meern, The Netherlands). RNA integrity was assessed on the 2100 Bioanalyzer (Agilent, Amstelveen, The Netherlands) using the RNA 6000 Nano Chip Kit (Agilent), and good quality RNA was used for gene expression analysis (RNA integrity number > 8.0). Gene expression analysis using the Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA) was performed as described previously (Theunissen et al., 2011). Quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were performed according to the manufacturer’s instructions and showed to be within acceptable limits for all chips.

**Data analysis and statistics.** Affymetrix CEL files were normalized using the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003) using RMAexpress (Bolstad et al., 2003). Raw and normalized data are available at ArrayExpress, http://www.ebi.ac.uk/arrayexpress/, under accession number E-TABM-1205. For probe to gene mapping, a custom Chip Description File (CDF) was used according to the assembly by de Leeuw (2008) (http://mad-db.science.uva.nl/~wdeleeuw/Hybrid/Annot/version6.html). Of the hybrid probe set definitions included in the custom annotation, 16,331 probe sets defined by the Brainarray custom CDF version 11 (http://brainarray.mbni.med.unich.edu/Brainarray/Database/CustomCDF) (Dai et al., 2005), and 4648 additional probe sets defined by Affymetrix chip annotation 26 were used in further analyses, giving a total of 20,979 probe sets. Probe sets for Affymetrix internal controls or probe sets that did not correspond to an Ensembl Gene ID were not used in further analyses. Statistical analyses were carried out using the R statistical software environment (http://www.R-project.org) using log-transformed values. For each gene, maximal fold change (FC) in gene expression between the
experimental groups was determined by comparing the average normalized signal values per group and was calculated as the maximum/minimum ratio. For compound toxicity effects, the treated samples were compared with the time-matched day 4 controls. Genes differentially expressed between any of the experimental groups (four concentrations and control) were identified by a one-way ANOVA. A significance threshold of false discovery rate (FDR) < 0.01 was used to select genes that were significantly differentially expressed due to compound exposure. Functional annotation and enrichment for gene ontology (GO) biological processes were studied using DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2007) and GenMAPP (Dahlquist et al., 2002). Significantly, enriched GO categories were based on a set criteria of permutation value (p < 0.01), Z-score (> 2), and genes changed within each specific GO gene category (> 5). GO terms with > 5000 genes and redundant GO terms were not included in the analysis. To evaluate quantitatively the changes within gene expression–linked GO gene categories, we used GO-Quant (Yu et al., 2006) to calculate the average absolute log2 FC associated with genes within each GO subset for each compound separately. Venn diagrams describing relations of significant gene expression and enrichment of GO terms between compounds were created using Venny (Oliveros, 2007). Hierarchical clustering of changes to control for all significantly differentially expressed genes (or subsets thereof selected based on Venn diagrams and GO terms) was performed in GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium), using Euclidean distance and Ward linkage.

RESULTS

Concentration-Dependent Effects of CYP, HEX, and VPA on Viability and Neurite Outgrowth

CYP, HEX, and VPA affected cell viability in a concentration-dependent manner (Fig. 1). Compound concentrations reducing cell viability to 80% (IC20) were 117.6μM for CYP, 19.8μM for HEX, and 2200μM for VPA. These data were used as a reference level for the highest concentration tested in the ESTn for both gene expression and neural morphological assessments.

On day 11, after a 72-h compound exposure from day 3 onward, both CYP and VPA significantly altered neurite outgrowth in the ESTn in a dose-responsive manner (Fig. 1). At the tested concentrations (0–25μM), HEX did not significantly impact neurite outgrowth at concentrations tested. However, in earlier performed concentration range-finding experiments, HEX did reduce neurite outgrowth at 100μM (data not shown), a highly cytotoxic concentration at which viability was reduced to approximately 10%.

Genes Regulated by CYP, HEX, and VPA in a Concentration-Dependent Manner

Compound-induced differential gene expression was compared between exposed mESC neural differentiation cultures and time-matched control cultures, using one-way ANOVA (FDR < 0.01). We identified 663, 761, and 3626 genes to be significantly regulated by CYP, HEX, and VPA, respectively (Fig. 2A). CYP regulated 0, 215, 134, and 281 genes with increasing concentrations tested. For Hex and VPA, these numbers were 0, 20, 87, 779 and 0, 147, 574, 2355, respectively. We observed 252 genes to be significantly regulated by all three compounds. In total, the expression levels of 3980 unique genes were concentration dependently regulated by any of the compounds (Fig. 2B). These genes were significantly differentially expressed after exposure to at least one of the compounds in at least one test compound concentration, as determined by one-way ANOVA including all tested concentrations plus control.

GO Terms Specifically Regulated by CYP, HEX, and VPA

To study the effects of compounds at the biological process level, GO terms significantly enriched by each of the three compounds were identified (Fig. 2C). Within the 663 genes observed to be significantly altered by CYP, 93 enriched GO terms were identified (Fig. 2C), including GO terms involved in general development (e.g., general, embryonic, CNS, organ), differentiation (cell, neuron), metabolism (sterol, lipid, isoprenoid, organic) transcription, biological regulation, cell cycle, and protein kinase pathways (Supplementary data). For the 761 genes regulated by HEX, only 28 GO biological processes were enriched (Fig. 2C), including GO terms related to development (e.g., general, embryonic, CNS, organ), metabolism (lipid, sterol, isoprenoid, organic), cell cycle, and transcription (Supplementary data). Within the 3626 genes regulated by VPA, 74 distinct GO terms were enriched (Fig. 2B), related to a wide array of processes, including development (general, embryonic, CNS, organ), differentiation (cell, neuron), metabolism (sterol, lipid, isoprenoid, organic, nucleic acid, and protein), cell cycle, cell death, oxidative stress, and localization (Supplementary data). Significance (p ≤ 0.01) and degree of significance (indexed as z*-log(p value)) for selected GO terms are shown in Figure 2D (complete list of GO-term significance values is provided in the Supplementary data), to illustrate degrees of GO-term enrichment. For example, steroid metabolic process is highly enriched in the triazoles compounds but only slightly enriched in VPA, whereas neuron development is highly enriched in VPA but slightly enriched in CYP and not enriched in HEX.

The 12 GO terms significantly enriched by all three compounds were related to embryonic morphogenesis, metabolic processes (including sterol and lipid metabolic process), and regulation of cell cycle (Fig. 2C). The 26 GO terms regulated by CYP and VPA but not HEX were involved in general development (e.g., developmental process, anatomical system development), nervous system development (neuron development, axon guidance), and cell differentiation and morphology (Fig. 2C). The 10 GO terms significantly enriched by CYP and HEX but not VPA were involved in steroid metabolic processes, embryonic development, and RNA processing (Fig. 2C). No GO terms were significantly enriched by both HEX and VPA, which were not also enriched by CYP.

Concentration-dependent effects of genes within selected enriched GO terms (embryonic morphogenesis, neuron development, regulation of cell cycle, programmed cell death, sterol metabolic process, and RNA processing) were examined for CYP, HEX, and VPA (Figs. 3A–C). Concentration-dependent changes in gene expression were associated with selected GO terms. For example, 21 genes associated with sterol metabolic process on average showed a concentration-dependent
increase in effect with CYP with a peak change in the high dose of 1.5 FC. Across selected functional groups, we observed genes associated with embryonic morphogenesis followed by genes associated with regulation of cell cycle and programmed cell death with all three compounds at all concentrations tested.

Effects on expression changes of single genes within the GO biological processes embryonic morphogenesis (Fig. 4A), neuron development (Fig. 4B), and sterol metabolic process (Fig. 4C) were visualized by hierarchical clustering. Genes significantly regulated (FDR < 0.01) by at least two of the three compounds are shown. In general, independent of significance, genes associated with embryonic morphogenesis were regulated by all three compounds in the same concentration-responsive manner in terms of directionality, with the exception of the highest concentration of VPA tested (1000 \( \mu \)M). For approximately 50% of embryonic morphogenesis genes regulated by two of the three compounds, VPA at 1000 \( \mu \)M resulted in differential directional regulated expression compared with the other exposure groups (Fig. 4A). Within the GO-term neuron development (Fig. 4B), a similar pattern of concentration-dependent regulation was observed across the three compounds, again with VPA at 1000 \( \mu \)M regulating genes in an opposite direction compared with lower doses and to the triazole compounds. Within the GO-term sterol metabolic process (Fig. 4C), the majority of the genes were concentration dependently upregulated in the triazole compounds but concentration dependently downregulated in VPA. For example, Cyp51, a gene, which is important in sterol metabolism but also in retinoic acid (RA) metabolism, was upregulated in a concentration-dependent way by the triazole compounds but downregulated by VPA.

**DISCUSSION**

In recent years, several additions to the EST developmental toxicity assay have been proposed, including the introduction of additional differentiation routes (Marx-Stoelting et al., 2009; Spielmann et al., 2006). We developed the complementary ESTn model to predict compound effects on the neuro-ectodermal lineage and investigated the effects of the neurodevelopmental toxicant MeHg (Theunissen et al., 2010). Studies describing molecular endpoints (Barrier et al., 2011; Seiler et al., 2004; Uibel et al., 2010) and transcriptomics techniques (van Dartel et al., 2009, 2010a,b) in the EST proved to be useful in further increasing the predictivity of the EST (Pennings et al., 2011; van Dartel et al., 2011b). Therefore, we studied the effects of MeHg over time in the ESTn using transcriptomics (Theunissen et al., 2011) and concluded that the most robust measurements can be achieved after 24-h treatment on day 4 of the standardized differentiation protocol. In the present study, concentration-dependent transcriptomic responses are studied by testing three known developmental toxicants cyproconazole (CYP), hexaconazole (HEX), and valproic acid (VPA) to further characterize the ESTn. The compounds were chosen such that we should be able to discriminate a triazole-specific response (CYP and HEX vs. VPA) and a neurodevelopmental toxicity-specific response (CYP and VPA vs. HEX), thus investigating whether the differential gene expression responses would display mechanistic specificity and add greater value than morphological assessments alone.

Relationships Between Compound Effects on Morphology and Gene Expression Changes

CYP, HEX, and VPA impacted cell viability with different potencies, with HEX being the most potent and VPA the least.
Compound effects on neurite outgrowth around the EB were concentration dependent for CYP and VPA. VPA severely reduced neurite outgrowth at concentrations without reduced viability, with approximately a 10-fold difference between the IC$_{20}$ for viability and the ID$_{20}$ for differentiation, showing that neurite outgrowth is a much more sensitive parameter compared with viability in this assay specifically for VPA. In contrast, only minor sensitivity differences were observed for CYP between compound effects on cytotoxicity and neurite outgrowth. We did not observe any sensitivity differences between cytotoxicity and neurite outgrowth for HEX. Associated with effects on cell viability and neurite outgrowth, we observed concentration-dependent effects on gene expression and corresponding functional GO terms. Despite using a similar range in potencies, VPA significantly altered the expression of approximately four times more genes than CYP and HEX, primarily driven by the greater gene expression response at the highest concentration tested, which was in line with the relatively large effect on neurite outgrowth in the morphological test.

Concentration-Dependent Compound Effects on GO-Term Enrichment

Analysis of GO terms regulated revealed major themes within genes significantly enriched by CYP, HEX, and VPA. In association with concentration-dependent effects on morphology, we observed common enrichment of GO terms related to development, metabolism, and cell cycle. Uniquely, GO terms related to cell differentiation and neuron differentiation were only significantly enriched by CYP and VPA and not by HEX. In addition, published data have also shown neurodevelopmental effects for CYP and VPA but not HEX, confirming the correlation between ESTn gene expression readout and existing in vivo and in vitro data (de Jong et al., 2011; Duncan, 2007; Machera, 1995). VPA specifically regulated programmed cell death and apoptosis-related GO terms, at a concentration that was not found to be cytotoxic in culture. VPA, which is also an HDAC inhibitor, is known to induce apoptosis through this mechanism (Mehnert and Kelly, 2007). Enrichment of apoptosis accompanied with no observed cytotoxicity in the cells was also observed earlier in the pluripotent murine embryonal carcinoma cell line after 24-h exposure to 1mM VPA (Jergil et al., 2009). In addition, effects on development-related GO terms were observed at concentrations at which no effects were observed on neural morphology or cell viability, implying that gene expression could be a more sensitive endpoint.

CYP, HEX, and VPA Disturb Genes Involved in Embryonic Development

Most GO terms were already regulated at the lowest concentration and concentration dependently upregulated in all three compounds, indicating that effects on the RNA transcript level can occur without inducing adverse effects on morphology. The direction of gene regulation within embryonic morphogenesis was comparable for the triazoles, signifying a comparable mode of action on the regulation of this GO term. However, VPA regulated gene expression of well-characterized developmental associated genes in an opposite direction compared with the triazoles (Fig. 4A), of which the majority is also important during neural development. These included Hox pathway–related genes (Hoxb4, Hoxc4, Hoxb5, Dlx5, and Msx1) (Bendall and Abate-Shen, 2000), genes of the transforming growth factor-β pathway (Lefty1) (Meno et al., 1996), early ectodermal formation and patterning (Fgf8) (Stavridis et al., 2010), early mesodermal formation (T) (Herrmann et al., 1990), and RA pathway–related genes (Rarb, Cyp26b1) (Duester, 2008; Yashiro et al., 2004). The different direction in which these genes are regulated by VPA as compared with the triazoles suggests a different mechanism of action, leading to comparable morphological outcomes in the ESTn.
CYP and VPA Responses Significantly Target Neural Development

Genes regulated within the neuron development GO-term showed a similar response for both the triazole compounds and VPA at the gene level, although only CYP and VPA significantly enriched this GO term. Genes regulated by both CYP and VPA included *Mtap2*, which is important for dendrite microtubule formation during neuron development (Farah and Leclerc, 2008), and the well-characterized *Pax6*, an important transcription factor during early ectodermal development (Callaerts et al., 1997). Compound effects on the genes within the neuron development GO-term observed in the ESTn corresponded with developmental toxic effects found in vivo. VPA is well known for its induction of spina bifida and NTDs (Duncan, 2007), and CYP was observed to increased incidence of hydrocephalus in rats (Machera, 1995), and furthermore, induced neural differentiation in the ESTc (de Jong et al., 2011). No effects of HEX on neural development have been observed in vivo (de Jong et al., 2011), which is in line with the absence of a significant regulation of the neuron development GO-term in this study.

CYP and HEX Increase Sterol Metabolic Process–Related Gene Expression, Whereas VPA Decreases Their Expression

As a main mechanism of action, triazoles inhibit the enzyme lanosterol 14-α-demethylase (Cyp51), which catalyzes the synthesis of ergosterol, an important protein for membrane integrity (Ghannoum and Rice, 1999). Valproic acid was observed to inhibit sterol synthesis in the developing rat brain (Bolanos et al., 1990), and more recent studies show that VPA causes a general downregulation of expression of genes encoding for enzymes early in steroidogenesis in the human carcinoma cell line H295R and murine Y1 adrenocortical cells (Brion et al., 2011; Gustavsen et al., 2009). All three compounds significantly regulated pathways involved in sterol synthesis in a concentration-dependent manner. For example, in the GO-term sterol metabolic process, gene expression was increased by both triazoles, however, VPA showed a decrease in the majority of the genes regulated in this pathway. CYP 51, for instance, was upregulated by CYP but downregulated by VPA. This indicates that although all three compounds have an effect on sterol metabolism, the mechanisms behind the effect are clearly different.

HDAC Inhibition Pathways Are Specifically Disturbed by VPA

A well-characterized mode of action of VPA is HDAC inhibition (Glaser et al., 2003), a mechanism by which VPA is thought to change overall gene expression (Göttlicher et al., 2001; Mehnert and Kelly, 2007; Phiel et al., 2001). A core set of 15 gene markers regulated by three HDAC inhibitors was described earlier (Glaser et al., 2003; Jergil et al., 2009). Of these 15 genes, 9 genes were significantly regulated (FDR < 0.01) by VPA in this study, whereas the triazoles CYP and HEX only regulated two of these genes (data not shown). Furthermore, direction of regulation by VPA was comparable to effects observed earlier in the pluripotent murine embryonal carcinoma cell line P19 (Jergil et al., 2009), showing that this VPA-specific main mechanism of action could be discerned in the ESTn.
CONCLUSION

The present study showed that differential gene expression provides a sensitive tool for studying the effects of chemicals on neural differentiation in the ESTn detecting effects at concentrations below those inducing morphological effects. Moreover, chemical-specific gene expression signatures can be discriminated, such as the triazole signature and the neuro-developmental toxicity signature in the present study, which allow mechanistic insight into the action of chemicals tested. This methodology therefore potentially offers significant added value to existing morphological effect assessment in ESTn and to the elucidation of the nature of developmental neurotoxicity of chemicals. Such mechanistic insights are instrumental in determining the extrapolation of adverse effects in alternative assays to human hazard assessment.

SUPPLEMENTARY DATA

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

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