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Research Article

Gene Expression Regulation and Pathway Analysis After Valproic Acid and Carbamazepine Exposure in a Human Embryonic Stem Cell-Based Neurodevelopmental Toxicity Assay

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ABSTRACT

Differentiating pluripotent stem cells *in vitro* have proven useful for the study of developmental toxicity. Here, we studied the effects of anticonvulsant drug exposure in a human embryonic stem cell (hESC)-based neurodevelopmental toxicity test (hESTn). During neural differentiation the cells were exposed, for either 1 or 7 days, to noncytotoxic concentration ranges of valproic acid (VPA) or carbamazepine (CBZ), antiepileptic drugs known to cause neurodevelopmental toxicity. The effects observed on gene expression and correlated processes and pathways were in line with processes associated with neural development and pharmaceutical mode of action. In general, VPA showed a higher number of genes and molecular pathways affected than CBZ. The response kinetics differed between both compounds, with CBZ showing higher response magnitudes at day 1, versus VPA at day 7. With this study, we demonstrated the potential and biological relevance of the application of this hESC-based differentiation assay in combination with transcriptomics, as a tool to study neurodevelopmental toxicity.

Key words: human embryonic stem cells; neural differentiation; valproic acid; carbamazepine; neurodevelopmental toxicity; transcriptomics

Pluripotent stem cells are employed in *in vitro* assays to study developmental toxicity *in vitro* (Estevan, 2011; Liu *et al.*, 2013; Marx-Stoelting *et al.*, 2009). Several protocols have been developed in which stem cells differentiate towards different cell linages *in vitro*, in which *in vivo* embryonic cell differentiation is mimicked (Buzanska *et al.*, 2009; Colleoni *et al.*, 2011; Desbordes *et al.*, 2008; Heuer, 1993, Itskovitz-Eldor *et al.*, 2000; Krug *et al.*, 2013; van Dartel *et al.*, 2009; Seiler and Spielmann 2011; Theunissen *et al.*, 2010). The mouse embryonic stem cell test (mEST) has proven a valid method to study the effects of compounds during cardiomyocyte differentiation, functioning as a parameter for developmental toxicity (Genschow *et al.*, 2004; Heuer, 1993; Seiler and Spielmann, 2010; Scholz *et al.*, 1999; van Dartel *et al.*, 2009). Furthermore, protocols in which mouse embryonic stem cells differentiate into other cell lineages, like neural and osteoblast differentiation, have been developed (de Jong *et al.*, 2012; Theunissen *et al.*, 2010). The original morphological endpoints take beyond a week to be expressed in these assays, and are difficult to quantify objectively. The inclusion of transcriptomics has led to more detailed read-out parameters, giving improved biological insight and reduced duration of the cell culture protocols (Krug *et al.*, 2013; Meganathan *et al.*, 2012; Steiner *et al.*, 2004; Theunissen *et al.*, 2011; van Dartel *et al.*, 2010).

In the last decade, the application of embryonic stem cells from human origin has become of great interest and is widely

studied resulting in various culture and differentiation protocols (Adler *et al.*, 2008; Axell et al. 2009; Colleoni *et al.*, 2011; Desbordes *et al.* 2008; Fathi *et al.* 2011; Iacovitti *et al.*, 2007; Krug *et al.*, 2013; Mehta *et al.*, 2008; Palmer *et al.*, 2013). The use of hESC as a starting point to study human developmental toxicity *in vitro*, in contrast to mESC, excludes the need for interspecies extrapolation. In an earlier study, we have developed a neural differentiation protocol using H9 hESC *in vitro* (hESTn) to study neurodevelopmental toxicity (Schulpen *et al.*, 2014a).

Here, we study the gene expression and pathway response of the hESTn in terms of compound- and concentration-specific effects on cell differentiation. The effects on gene expression are also studied on the basis of gene ontology (GO) and pathway analysis and compared to *in vivo* effects, in order to evaluate biological relevance of gene expression responses. We exposed the hESTn for either 1 or 7 days to increasing concentrations of valproic acid (VPA) and carbamazepine (CBZ) as model compounds. Both compounds are pharmaceuticals used as antiepileptic drugs and are known to cause neurodevelopmental toxicity (Alsdorf and Wyszynski 2005; Afshar *et al.*, 2010; Brunn *et al.* 2014; Hill *et al.*, 2010; Matalon *et al.*, 2002). Furthermore, in earlier *in vitro* studies, it was demonstrated that these compounds affected myocardial and neural mESC differentiation (Theunissen *et al.*, 2012a, 2013) and affected genes involved in hESC pluripotency and neural differentiation. However, in the latter study the effects on hESC differentiation were assessed for 7 genes only, using Q-PCR. Here, we expanded the study of VPA and CBZ effects in hESTn by whole-genome transcriptomics array analysis.

MATERIALS AND METHODS

Human embryonic stem cell culture. H9 hESC (WA09-DL11, WiCell, Madison, Wisconsin) were cultured as described in Schulpen *et al.* (2014b). Briefly, the hESC were routinely cultured on mytomycin mitotically inactivated mouse embryonic fibroblast at 37°C in hESC medium, containing DMEM-F12, supplemented with 20% Knock Out Serum Replacement (KOSR), 1 mM L-Glutamine, 0.5% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin, 1% nonessential amino acids, 0.1 mM β-Mercaptoethanol and 0.2 μg/ml fibroblast growth factor-basic (bFGF). hESC were passaged 2–3 per week.

Neural differentiation. hESC cells were differentiated as described in Schulpen *et al.* (2014a) (Fig. 1). Briefly, hESC were enzymatically dissociated after incubation with Collagenase IV and transferred to bacterial culture dishes containing hESC culture medium (Fig. 1A and 1B). Within 4 days, the cells formed cell aggregates, which were transferred to Poly-D-Lysine (PDL)/Laminin-coated cell culture dishes containing DMEM-F12 supplemented 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin, 1.5 mM L-glutamine and 10% insulin, transferrin, and Selenium (ITS) premix (Fig. 1C and 1D). The cell aggregates attached to the bottom of the dishes and were cultured for 3 days. At day 7, the ITS medium was replaced by neurobasal medium supplemented with 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin, N-2- and B27 premix (Fig. 1E). For the morphological end point (not used in this study), the cells were cultured until day 11, with a medium refreshment at day 9 (Fig. 1F).

Exposure. Differentiating cell cultures were exposed for either 1 or 7 days (indicated with a green bar in Fig. 1) to either 0.1 ($n = 2$), 0.33 ($n = 6$) or 1 mM ($n = 6$) VPA (Sigma–Aldrich, CAS 1069-66-5), or 0.033 ($n = 2$), 0.1 ($n = 6$), or 0.33 mM ($n = 6$) CBZ (Sigma–Aldrich, CAS 298-46-4), starting after the hESC were dissociated (day 0). These concentrations had been shown to produce less than 30% viability loss in a 5-day cell viability assay (Schulpen *et al.*, 2014b). VPA and CBZ were dissolved in medium and 0.25% Dimethylsulfoxide (DMSO), respectively. Unexposed control samples were collected at day 0 ($n = 6$), 1 ($n = 5$), 4 ($n = 4$),7 ($n = 6$), and 9 ($n = 2$). The control samples taken at days 1 and 7 served as time-matched controls for VPA exposure after 1 and 7 days, respectively. At days 1 and 7 also samples cultured with 0.25% DMSO ($n = 6$) were collected serving as a time-matched controls for 1 and 7 days CBZ-exposed groups, respectively. Together with medium replacement at days 0 and 4, VPA or CBZ were added back to the culture medium at the appropriate concentrations.

RNA extraction and processing. Cells ready for RNA extraction (indicated in Fig. 1 as 'RNA extraction Cont. + exp'), controls and exposed, respectively, were directly collected in RNA protect (Qiagen) and subsequently stored at −20°C. Before RNA extraction, the cells in RNA protect were thawed, centrifuged, and the RNA protect was discarded. The RNA was extracted using the QiaCube and RNeasy mini kit (Qiagen) including an DNase incubation step, following the manufactures manual. The extracted RNA was stored at −80°C.

The concentration of the RNA samples was measured using the Nanodrop (Thermo Scientific).

Microarray analysis. RNA samples were randomized and further processed for hybridization to Affymetrix HT HG-U133 + PM plates at the Microarray department (MAD) of the University of Amsterdam, The Netherlands. Amplification, labeling and hybridization was performed according to Affymetrix protocols, using an automated Affymetrix Genechip console. For each individual sample, 100 ng RNA was used for the biotin-labeling reaction. The labeled cRNA was fragmented and hybridized to the Affymetrix HT HG-U133 + PM plates. After staining, the plates were scanned with Genechip HT array plate scanner and analyzed with Affymetrix HT software suite. The microarray data is accessible at NCBIs Gene Expression Omnibus, under GEO accession number GSE 64123.

Data analysis and processing. Quality control and normalization of Affymetrix CEL files were performed using the ArrayAnalysis website (http://www.arrayanalysis.org/) (Maastricht University, The Netherlands) (Eijssen *et al.*, 2013), using the Robust Multichip Average (RMA) algorithm (Irizarry *et al.*, 2003) and MBNI custom CDF version 14 (Dai *et al.*, 2005). Two samples were discarded after quality check: CBZ 0.33 mM days 1 and 7. Normalized data was Log2 transformed. Of each sample, the average fold change (FC) was calculated using R. This resulted in expression ratio values of the cultures exposed to either VPA or CBZ compared to the time-matched control samples. Differentially expressed genes were identified using a 1-way Anova (OWA) analysis with a significance threshold of $P \leq .001$ and FDR $\leq 5\%$ using R. Overlapping genes were calculated and displayed as Venn diagrams using Venny (Oliveros, 2007).

Principal component analysis (PCA) was used to visualize samples in relation to time (differentiation) and compound exposure (concentration) dependent effect using R. Input for the PCA analysis consisted of expression data for the union of genes differentially expressed over time (between 3 control time points) and those differentially expressed across all concentrations either after 1 or 7 days of exposure to either VPA or CBZ.

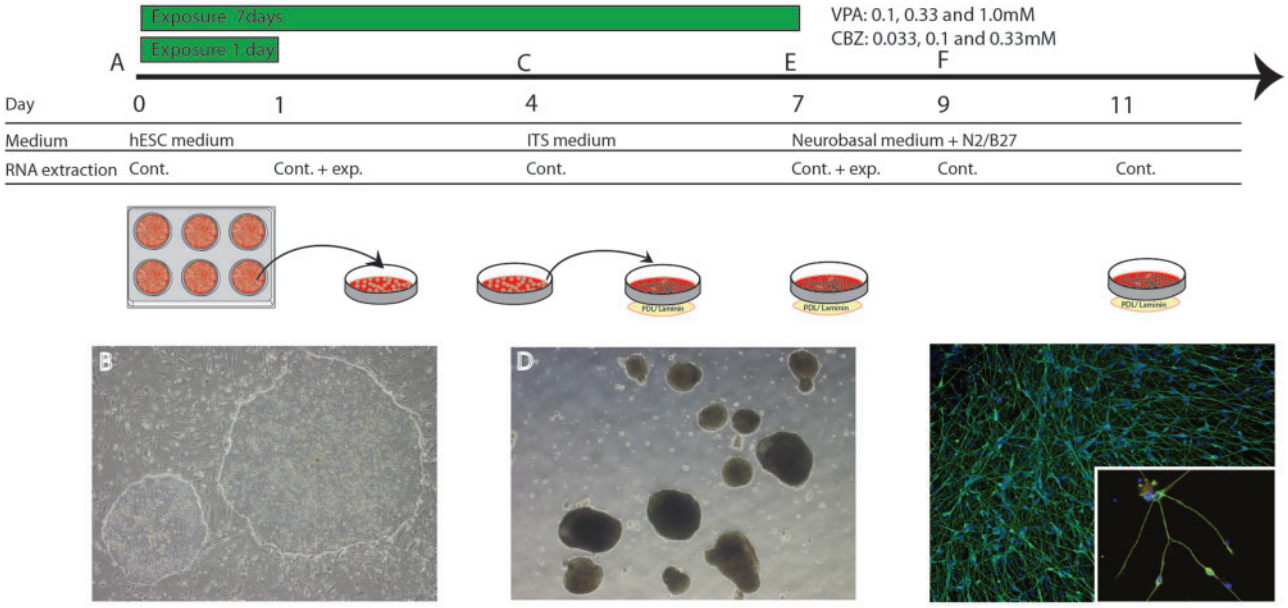

FIG. 1. Method and study design. Schematic representation of study design. For detailed description of stages (A–F), see Materials and Methods.

Significantly affected pathways and GO terms were identified with Tox-profiler (Boorsma *et al.*, 2005), on the basis of whole genome expression ratios after 7 days. Significance was calculated based on T- and E-values. The T-value was obtained by a T-test between the expression changes for a defined gene set compared to all other genes. The E-value was the associated 2-tailed *P* value with Bonferroni correction for the number of gene sets tested (Boorsma *et al.*, 2005). To study and compare the effect on a particular GO-term caused by each individual concentration both after 1 and 7 days of VPA and CBZ exposure, the genes involved in individual GO terms were selected and the average FC for each sample was calculated. Pathway analysis of significantly differentially expressed genes after 1 day of either VPA or CBZ exposure was performed using Metacore (Thomson Reuters, Philadelphia, Pennsylvania) (https://portal.genego.com). For each significant gene, the average FC was calculated, both after 1 or 7 days of exposure.

RESULTS

Differentially Expressed Genes

After 1 and 7 days of exposure to VPA, 3696 and 5233 genes were significantly regulated ($P \leq .001$ and FDR $\leq 5\%$) across all concentrations, respectively, with 2234 of these genes regulated at both time points (Fig. 2). After 1 and 7 days of exposure to CBZ, 682 and 1090 genes were significantly regulated across all concentrations, respectively, with 112 of these genes regulated at both time points. Moreover, the vast majority of VPA-regulated genes were not regulated by CBZ, and time- and compound-dependent differences in responsive genes overlapping among experimental groups were apparent (Fig. 2).

PCA Analysis

Time- and compound-concentration dependent global gene expression changes were visualized in PCA plots (Figs. 3A–D). We used days 0, 1, and 4 control gene expression data to compare the effects of 1 day exposure (Fig. 3A and B), and days 4, 7, and 9 control gene expression data for comparing the effects of 7-day exposure (Fig. 3C and D). These figures show that exposure caused compound- and concentration-dependent

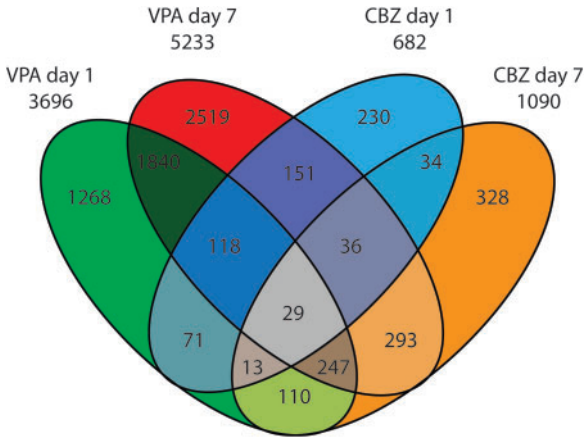

FIG. 2. Venn diagram of statistically significant gene expression changes ($P \leq .001$ and FDR $\leq 5\%$) after either 1 or 7 days of exposure to either VPA or CBZ.

deviation from the time-dependent differentiation track, both after 1 and 7 days of exposure. Moreover, the inserted Venn diagrams in Figure 3 show that in each case both anticonvulsant drugs affected genes that were regulated time dependently as well as genes that were not regulated time dependently. As an example, valproate after 1 day of exposure regulated 698 time-regulated genes plus 2998 time-independent genes, whereas 1152 time-regulated genes were not affected by valproate exposure (Fig. 3A). Similar patterns of common and unique gene expression changes between time and exposure were observed for the other exposures applied (Figs. 3B–D).

Tox Profiler Gene Ontology Analysis

Using Tox-profiler, analysis of the complete dataset of compound-induced gene expression changes revealed 126 GO gene groups significantly regulated in at least one of the experimental groups in this study (T value ≥ 3.5 and $E \leq 0.05$) (Fig. 4). Both anticonvulsant drugs showed common and specific GO terms regulated, revealing compound-specific characteristics of the gene expression response. We further analyzed the extent of gene expression response within 3 GO terms regulated by VPA but not

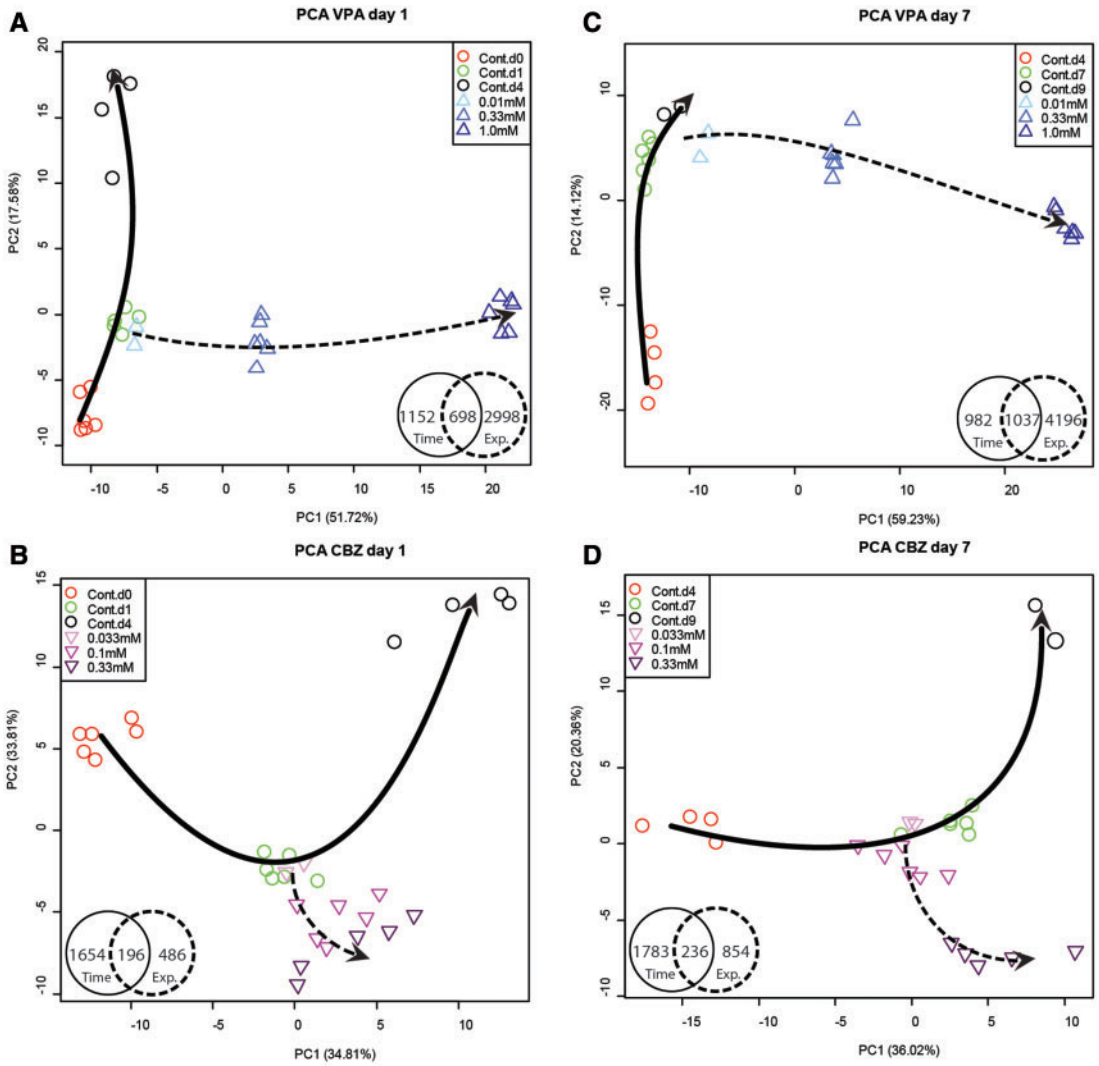

FIG. 3. PCA plots of global gene expression response after 1 day of either VPA (A) or CBZ (B) exposure compared to control samples at days 0, 1, and 4 and response after 7 days of either VPA (C) or CBZ (D) exposure compared to control samples at days 4, 7, and 9.

CBZ, 'ion transport', 'synapse', and 'axon', 3 GO terms regulated by CBZ but not VPA, 'angiogenesis', 'cholesterol homeostasis' and 'anterior/ posterior pattern formation', and 2 GO terms regulated both by VPA and CBZ, 'calcium ion binding', and 'chromatin modification' (Fig. 5). This analysis shows that the percentage of genes responding significantly ($P < .001$) within GO terms was in all cases higher after VPA as compared to CBZ exposure, even for those GO terms which responded significantly to CBZ exposure only. The absence of significance of the VPA response in these CBZ-regulated GO terms is most likely due to the abundance of gene expression changes induced by VPA in general, increasing the threshold for statistical significance of VPA-mediated regulation for individual GO terms. Also when comparing equimolar concentrations (0.33 mM) only, the maximal observed response to VPA was higher than for CBZ in these GO terms. Furthermore, whereas the VPA response within GO terms increased between days 1 and 7 of exposure, CBZ generally tended to show a similar or even higher response level at day 1 as compared to day 7.

Metacore Pathway Analysis

Using Metacore pathway analysis, significantly differentially expressed genes after 1 day of VPA and CBZ exposure were linked to 61 and 31 significantly regulated pathways ($P \leq .001$) (data shown in Supplementary Table 1). Based on literature, 8 pathways (shown in Table 1 and Supplementary Fig. 1) involved in pharmacologic mechanism of action, (neuro) development, and toxicity, were selected for further analysis. VPA affected a higher number of genes in each of these pathways and showed a related higher level of significance, compared to CBZ exposure.

DISCUSSION

We studied the global gene expression response of a hESC-based neural differentiation assay to 2 antiepileptic pharmaceuticals, VPA and CBZ. These anticonvulsant drugs are known to cause neurodevelopmental toxicity in experimental animals and man. In this study, differentiating hESC cells were exposed for 1 or 7 days to VPA or CBZ. The exposure duration was based on earlier studies. In Schulpen *et al.* (2014a), we demonstrated significant differentially expression of 2 genes involved in stem cell renewal and maintenance of pluripotency (Pou5F1 and Nanog) with in parallel 5 genes involved in neurogenesis (βIII-tubulin, Neurogenin1, Reelin, MAPt, and MAP2) after 7 days of

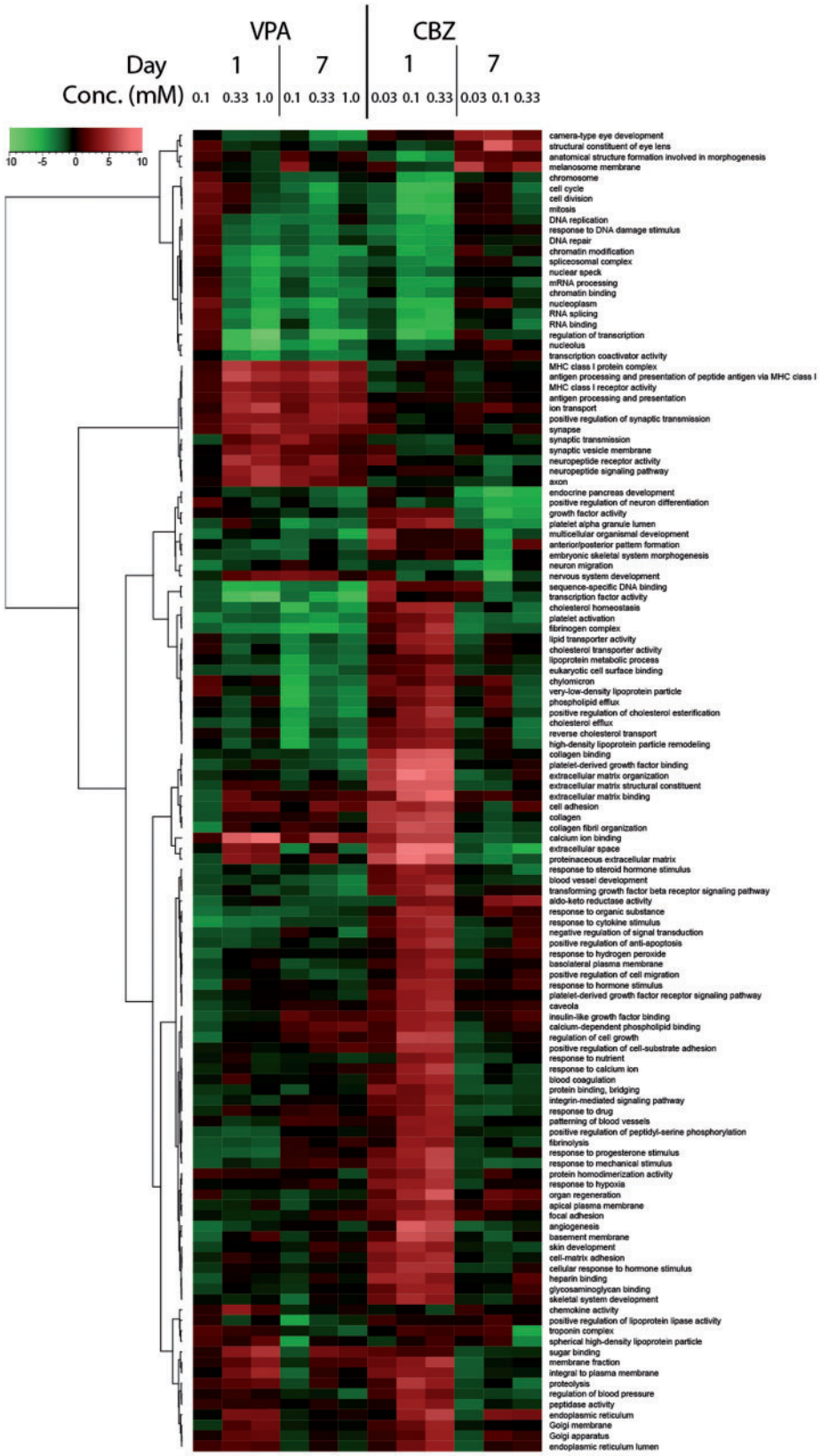

FIG. 4. Comparative heat map of relative expression of GO terms significantly regulated after at least one exposure scenario, as compared to time-matched untreated control values.

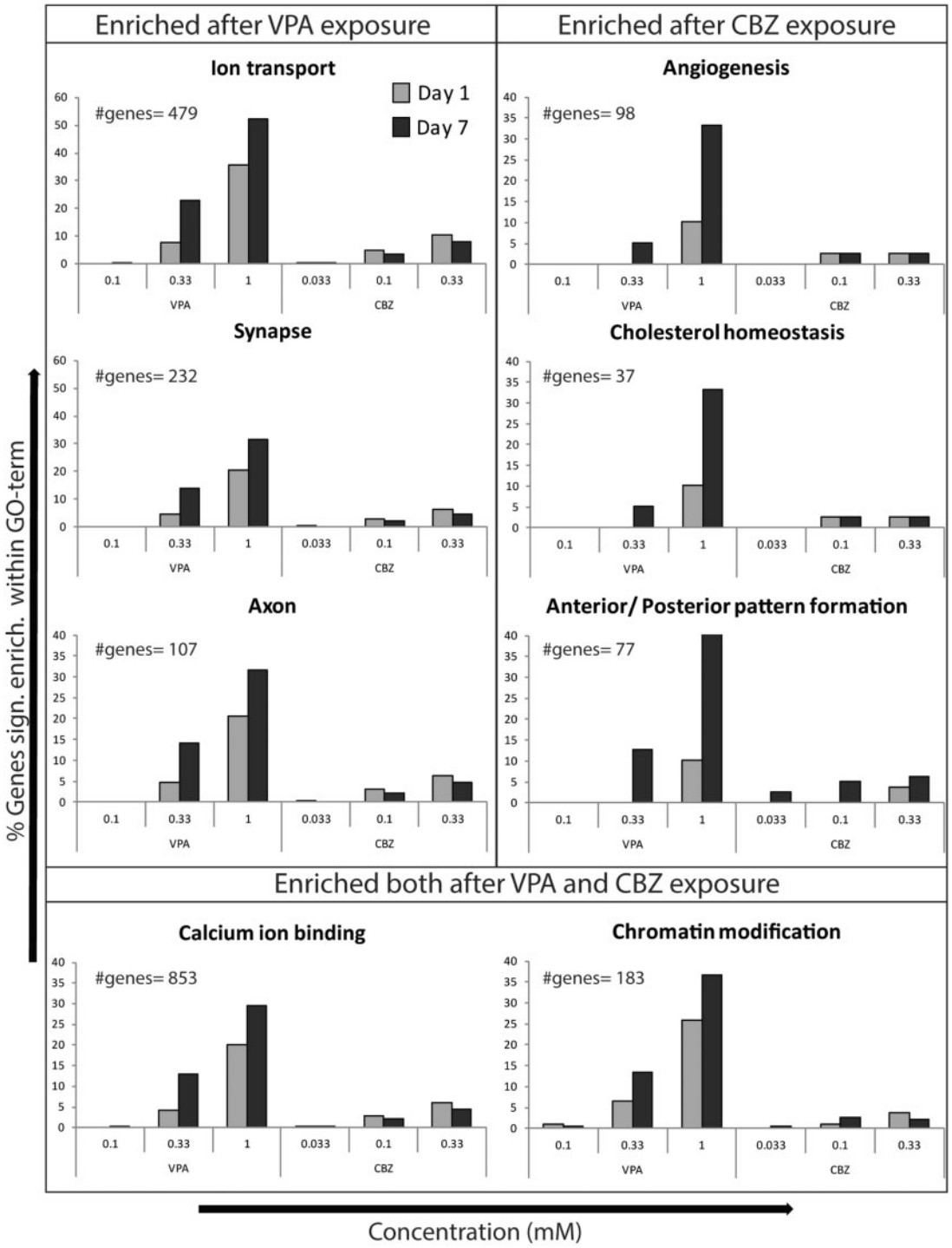

FIG. 5. Selected responses of GO-term significantly enriched in T-profiler after either VPA exposure (A), CBZ exposure (B), or in both experiments (C), expressed as. Percentage of genes within terms significantly differentially regulated.

differentiation. However, in line with earlier studies indicating that early gene expression responses are most specific for revealing compound mechanism of action (Balmer *et al.*, 2014) an early time point during differentiation may be preferable. In addition, a fast read-out system is preferable for an *in vitro* screening test. Selected exposures were close to the pharmaceutical systemic concentration range, which is between 0.3 and 0.8 mM and 0.02 and 0.05 mM for VPA and CBZ, respectively, (Medscape, 2013, 2014). The tested concentrations did not affect cell viability by more than 30%, as determined in (Schulpen *et al.*, 2014b).

Although the mechanisms involved in its therapeutic mode of action are not fully understood (Castro *et al.*, 2005; Rosenberg, 2007), several mechanisms of VPA have been described. The major mechanisms of pharmacological action involve the enhancement of the γ-aminobutyric acid-mediated (GABA) neurotransmission and inhibition of voltage-gated sodium channels by which high-frequency firing of neurons is inhibited

TABLE 1. Pathways Significantly Enriched After VPA Exposure and/or after CBZ Exposure, Involved in Neurogenesis or Pharmaceutical Mechanism of Action, Showing the Total Number of Genes Described Within the Pathway and the Number Statistically Significantly Regulated After Exposure

Pathway	Number of Genes in Pathway	Number of Genes Regulated After Exposure	Over-Representation *P* value	FDR	# of Genes Regulated After Exposure	Over-Representation *P* value	FDR
Development_WNT signaling pathway. Part 2	53	17	5.70E−05	3.73E−03	9	1.80E−05	1.11E−03
Development_TGF-beta receptor signaling	50	16	9.67E−05	5.49E−03	9	1.10E−05	9.28E−04
Development_Oligodendrocyte differentiation from adult stem cells	51	15	4.47E−04	9.28E−03	4	5.93E−02	2.43E−01
Neurophysiological process_ACM regulation of nerve impulse	46	14	4.68E−04	9.49E−03	1	7.42E−01	8.23E−01
Development_Role of CDK5 in neuronal development	34	11	1.07E−03	1.66E−02	1	6.32E−01	7.78E−01
Regulation of GSK3 beta in bipolar disorder	45	13	1.27E−03	1.89E−02	5	9.27E−03	9.35E−02
Neurophysiological process_Constitutive and regulated NMDA receptor trafficking	63	16	1.69E−03	2.12E−02	1	8.44E−01	8.67E−01
Signal transduction_Erk interactions: inhibition of Erk	34	10	3.94E−03	3.44E−02	1	6.32E−01	7.78E−01

(Brunn *et al.*, 2014; Castro *et al.*, 2005; Nanau and Neuman, 2013; Rosenberg, 2007). Furthermore, VPA inhibits histone deacetylase (HDAC) enzyme activity, stimulates mitogen-activated protein kinase (MAPK) (Castro *et al.*, 2005) and affects Wnt/b-catenin and the ERK Pathway (Rosenberg, 2007). The precise involvement of the latter 2 processes in therapeutic effects is unknown (Rosenberg, 2007). Additionally, VPA is known to be neuroprotective through an HDAC inhibitory effect and ERK pathway involvement (Biermann *et al.*, 2010). However, the dosimetry underlying neuroprotective versus neurotoxic effects is unclear (Brunn *et al.*, 2014). VPA exposure during pregnancy has been associated with a variety of developmental anomalies, neural tube defects, spina bifida, cleft lip and palate, cardio vascular abnormalities, developmental delay, limb defects, autism, and bipolar (Alsdorf and Wyszynski, 2005) disorder. The mechanisms involved in teratogenicity are not clear. However, the inhibitory effect of VPA on HDAC may disturb normal gene transcription during fetal cell proliferation and differentiation, which is initiated by hyperacetylation of the DNA histones by histone acetyltransferase enzymes (HATs) which results in unraveling chromatins, facilitating transcription (Lloyd, 2013).

CBZ is a commonly prescribed anticonvulsant drug, which acts via multiple mechanisms like blocking and potentiation of cation channels (Ambrosio *et al.*, 2000; Beutler *et al.*, 2005). Furthermore, it modulates the release, uptake, and receptor binding of neurotransmitters (Beutler *et al.*, 2005). Many animal studies have shown CBZ effects on the developing embryo. However, the results of studies in pregnant women are conflicting, because some describe an increased rate of anomalies, while others did not find and increase. Malformations associated with maternal use of CBZ include neural tube defects, cleft palate, cardiac- and urinary tract anomalies (Afshar *et al.* 2010; Matalon *et al.*, 2002; Ornoy 2006). The mechanism underlying CBZ toxicity is not known. However, CBZ is an effective HDAC inhibitor (Beutler *et al.*, 2005), which may explain the developmental anomalies.

The Venn diagram analysis revealed that both compounds showed time-dependent changes in gene expression, including common genes as well as unique genes regulated by either compound. The PCA plot confirmed these findings, with additional concentration-dependent effects, at the level of regulation of the entire genome. (Neuro-) developmental GO terms, like 'Embryonic and Tissue Morphogenesis' were observed in the overlapping field in the Venn diagram between VPA and CBZ exposure after 7 days and the overlapping field between VPA days 1 and 7, like 'Axonogenesis' and 'Neuron Projection Morphogenesis'. Furthermore, the overlapping field between VPA exposure after days 1 and 7 included pharmacological mode of action associated GO terms like, 'Chromatin- and Histone Modification'. Overlapping fields between CBZ after 1 and 7 days of exposure resulted in no significant GO-term enrichment. The heat map of GO terms significantly regulated by at least 1 compound (Fig. 4) extends the observation that the VPA response was more pronounced in terms of both the number of genes regulated and the magnitude of the responses. Although this is in part related to differences in molar concentration tested, the concentration-dependent gene regulation within selected GO terms (Fig. 5) confirms that VPA induces a stronger response than CBZ at equimolar concentrations. Moreover, similar findings have occurred in other systems in which the gene expression responses of VPA and CBZ showed comparable differences (Hermsen *et al.*, 2013; Theunissen *et al.* 2012b). This is in line with congenital malformations found *in vivo* after exposure, in which CBZ seems to be less teratogenic compared to VPA (Jentink *et al.*, 2010). Interestingly, Figure 4 also revealed differences in magnitude of responses with time of exposure between both drugs. VPA tended to show a higher response at day 7, whereas CBZ was more effective at day 1. Given the variety of GO terms regulated overall, the specificity of the response did not seem to differ between time points. Literature data indicate that early gene expression responses are more specific than later ones, the latter primarily revealing

nonspecific downstream effects (Balmer *et al.*, 2014). Time-dependent effects may also be developmental stage dependent as exposures occur over several important developmental windows. These findings indicate that the study design, including the exposure timing and duration, may affect the outcome of *in vitro* test systems.

Beyond the GO term library, global molecular pathways such as collected in Metacore offer a more specific tool for the analysis of gene expression responses. Although these pathways have not been fully validated, they can give insight into the interpretation of individual genes related to pharmacological action and developmental neurotoxicity of the tested compounds. Metacore clearly detected specific pharmacological and neurodevelopmental pathways affected by both compounds, and again the response to VPA was more pronounced than that to CBZ. These findings are generally in line with the abundant knowledge on VPA modes of action versus limited knowledge base on CBZ mechanisms of action. It is of interest to note the regulation of WNT and TGFB signaling by both compounds, two processes that are of crucial importance in neurodevelopment. The WNT signaling pathway plays an important role in embryonic development involved in signaling during mesoderm, neuroectoderm and body axis formation (Caroline R. Kemp, 2007). Furthermore, the Wnt/β-catenin signaling plays a major role in maintaining self-renewal as well as in regulating ESCs differentiation. In our analysis, we observed a significant down regulation of Tcf/Lef genes in the Wnt/β-catenin signaling pathway, which enhances self-renewal and results in differentiation resistance in mouse ESC (Atlasi *et al.* 2013). The TGFβ signaling pathway controls cellular processes including cell proliferation, differentiation and migration (Wrana *et al.* 1994, Shi and Massague 2003). The ERK-pathway, involved in cell growth, proliferation and cell survival (Junttila *et al.* 2008), and an important target in neuronal signal transduction and involved in neuronal maturation and survival, known to be activated by VPA exposure (Castro *et al.* 2005), involved in cell growth, proliferation and cell survival (Junttila *et al.* 2008), was found to be significantly enriched after VPA exposure. CBZ only regulated 1 gene involved within the ERK pathway. Within neurons, activation of NMDA receptors leads to stimulation of Calmodulin regulated Calcineurin A activation which subsequently activates STEP, which is expressed in several neuronal cell types (Paul *et al.* 2003). Activated STEP limits the duration of ERK activity (Paul *et al.* 2003), regulating the duration of ERK signaling in neurons. After exposure to VPA we found an upregulation of the Calmodulin/Calcineurin A/STEP genes. Further study into these signaling pathways and their response to anticonvulsant drugs in developmental systems may reveal common mechanisms of neurodevelopmental toxicity for this class of drugs. Both processes 'Neurophysiological process_ACM regulation of nerve impulse' and 'Neurophysiological process_Constitutive and regulated NMDA receptor trafficking', involved in neurotransmission, were both affected after VPA exposure, resulting in 14 and 16 genes significantly regulated, respectively. After CBZ exposure only 1 gene (IP3 receptor) was regulated in both processes. Interestingly, although it is known that these antiepileptics will act on glutamatergic neurotransmission, reducing fast excitatory neurotransmission (Kwan *et al.*, 2001) after VPA exposure, all genes regulated in theses pathways, except 1 (PKA-reg) were upregulated, which is in contrast to what was expected. The biological significance of regulation of genes in this pathway is limited by the fact that full glutamatergic cell maturation is not likely in this short term cell differentiation model. VPA and CBZ affected 13 and 5 genes within the 'regulation of GSK3 beta in bipolar disorder' pathway, respectively. It has been suggested that in bipolar disorder, mood stabilizers including VPA, CBZ and Lithium also act by the down regulation of glutametergic signaling (Basselin *et al.*, 2008), in which GSK3 beta plays and important role and is a molecular target. (Gurvich and Klein, 2002).

Taken together, this study shows that the hESTn assay allows the investigation of common and unique genome-wide gene expression changes and their functional coupling to specific pharmacological and neurodevelopmental regulatory pathways. The human origin of the cells circumvents the need for interspecies extrapolation. Although a stronger effect on gene expression was observed after 7 days of exposure, the brief exposure of 1 day already reveals neurodevelopment-specific gene expression responses. This allows for a brief culture duration and increased throughput. Human stem cell based *in vitro* tests such as the hESTn can assist in a screening situation to prioritize compounds for further development as potential drugs.

SUPPLEMENTARY DATA

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

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