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Distinct gene expression responses of two anticonvulsant drugs in a novel human embryonic stem cell based neural differentiation assay protocol

Sjors H.W. Schulpen [a,b,*], Esther de Jong [a,b], Liset J.J. de la Fonteyne [a], Arja de Klerk [a], Aldert H. Piersma [a,b]

[a] Laboratory for Health Protection Research, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands
[b] Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands

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ABSTRACT

Hazard assessment of chemicals and pharmaceuticals is increasingly gaining from knowledge about molecular mechanisms of toxic action acquired in dedicated *in vitro* assays. We have developed an efficient human embryonic stem cell neural differentiation test (hESTn) that allows the study of the molecular interaction of compounds with the neural differentiation process. Within the 11-day differentiation protocol of the assay, embryonic stem cells lost their pluripotency, evidenced by the reduced expression of stem cell markers Pou5F1 and Nanog. Moreover, stem cells differentiated into neural cells, with morphologically visible neural structures together with increased expression of neural differentiation-related genes such as βIII-tubulin, Map2, Neurogin1, Mapt and Reelin. Valproic acid (VPA) and carbamazepine (CBZ) exposure during hESTn differentiation led to concentration-dependent reduced expression of βIII-tubulin, Neurogin1 and Reelin. In parallel VPA caused an increased gene expression of Map2 and Mapt which is possibly related to the neural protective effect of VPA. These findings illustrate the added value of gene expression analysis for detecting compound specific effects in hESTn. Our findings were in line with and could explain effects observed in animal studies. This study demonstrates the potential of this assay protocol for mechanistic analysis of specific compound-induced inhibition of human neural cell differentiation.

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1. Introduction

Humans are exposed daily to chemicals that lack information concerning toxicity and their potential health hazards. For instance, under the REACH legislation for chemical safety in Europe, reproductive and developmental toxicity testing is only mandatory at high tonnage production levels. These tests are costly and require an estimated 65% of all animal use under REACH (der Jagt et al., 2004). There is a high need for efficient predictive alternative test methods, in order to inform about reproductive and developmental toxicity at lower production levels, and to reduce animal use in toxicological hazard assessment. During the last decades, much research has been performed toward the development of *in vitro* methods, which can contribute to the reduction of animal use in toxicological testing. For developmental toxicity testing several *in vitro* systems have been developed, varying from whole embryo cultures to assays based on cell lines (Spielmann, 2009).

The use of embryonic stem cells as a corollary of cell differentiation in the embryo is practical since they are relatively easy to culture, have a self-renewal capacity and can be cultured in undifferentiated state. Furthermore, they can differentiate into cell lineages originating from all three germ layers (Thomson et al., 1998), which make them suitable to study early developmental processes at the cellular level. In the embryonic stem cell test (EST), developed in 1993 by Heuer et al. (1993), Scholz et al. (1999), ES-D3 mouse embryonic stem cells differentiate into contracting cardiomyocytes. Embryotoxicants exert an inhibitory effect on the differentiation process, resulting in a concentration dependent inhibition of contracting cardiomyocyte foci formation.

Abbreviations: CBZ, carbamazepine; CM, culture medium; EST, embryonic stem cell test; FBS, Fetal bovine serum; hESC, human embryonic stem cells; hESTn, human neural embryonic stem cell test; ITS, insulin transferrin selenium; KOSR, knockout serum replacement; MEFs, mouse embryonic fibroblasts; mESC, mouse embryonic stem cells; mESTn, mouse neural embryonic stem cell test; MMC, Mitomycin C; PDL, Poly-D-Lysine; VPA, valproic acid.

* Corresponding author at: Laboratory for Health Protection Research, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands. Tel.: +31 30 274 2471.

E-mail address: sjors.schulpen@rivm.nl (S.H.W. Schulpen).

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More recent studies have enhanced the mechanistic readout of EST by incorporation of transcriptomics (van Dartel et al., 2010; van Dartel and Piersma, 2011) and by the establishment of differentiation culture protocols for several other differentiation routes, e.g. neural and osteoblast differentiation (de Jong et al., 2012; Theunissen et al., 2010). Compound exposure affected gene expression already after 24 h of exposure and resulted in compound- and concentration dependent responses (van Dartel et al., 2009). Gene expression analysis enhanced mechanistic insight into differentiation inhibition caused by compound exposure (van Dartel et al., 2009, 2011a,b).

The classical EST assays are based on murine embryonic stem cells, which are not completely representative for human cells. Current trends in toxicological hazard assessment move toward human based test systems (Mori and Hara, 2013; Liu et al., 2013; Buzanska et al., 2009; Krug et al., 2013) to facilitate human hazard and risk assessment. The application of established human embryonic stem cell lines can be instrumental in avoiding interspecies extrapolation and would result in reduced animal use in hazard assessment. Several human stem cell based differentiation assays have been developed, either for regeneration and replacement therapies (Iacovitti et al., 2007; Schulz et al., 2003), to study mechanisms involved in neurogenesis (Iacovitti et al., 2007; Fathi et al., 2011; Talens-Visconti et al., 2011; Ebert et al., 2013; Liu et al., 2013), or for neurodevelopmental toxicity testing (Buzanska et al., 2009; Schulz et al., 2003; Adler et al., 2008a; Colleoni et al., 2011; Hoelting et al., 2013). The assays differed widely in terms of culture method, with single- and multiple step replating approaches, and including rosette, neurosphere or embryoid body formation. Differentiation time differed between tests from a few days to several weeks, depending on the end-points assessed. In addition, different culture well coatings, additives and culture conditions have been employed. In the present study we developed a straightforward and relatively fast differentiation method in which pluripotent WA09 (H9) human embryonic stem cells differentiate into neural cells. In this method hESC differentiated through a minimal number of culture steps and few culture medium additives. Thus, a more spontaneous differentiation was achieved. Differentiation was studied using immunostaining, in which stem cells were stained with anti-SSEA4 and neural differentiation was evidenced by neuron specific anti βIII-tubulin staining. RT-PCR analysis was used to study the expression of mRNA transcripts associated with stem cell renewal and maintenance of pluripotency using Pou5F1 and Nanog. Gene expression involved in neurogenesis was studied with Neurogin1 and Reelin and neurons were evidenced by the expression of βIII-tubulin, MAP2 and MAPt.

To study the effectiveness of this model as an *in vitro* method to evaluate neurodevelopmental toxicity, the effects of valproic acid (VPA) and carbamazepine (CBZ) on neural differentiation were studied. Both VPA, an anticonvulsant and therapeutic drug for bipolar disorder (BPD) Gurvich and Klein (2002) and CBZ, an anticonvulsant drug (Kou et al., 2011), are known to cause neurodevelopmental toxicity *in vivo* (Jentink et al., 2010; Ornoy, 2006), VPA exposure during pregnancy can cause neural tube defects, spina bifida aperta, cleft palate and limb defects (Gurvich and Klein, 2002; Robert and Guibaud, 1982). CBZ can cause spina bifida, cardiovascular anomalies, cleft palate, skeletal- and brain anomalies (Ornoy and Cohen, 1996; Jones et al., 1989; Rosa, 1991). Earlier whole genome array gene expression studies in mouse EST have shown abundant gene expression responses of VPA with many thousands of genes responding, whereas CBZ, given at equipotent concentrations as to morphological cell differentiation inhibition, showed a relatively limited gene expression response (Theunissen et al., 2012a,b). These results already indicated that differential gene expression analysis may reveal compound-specific effects at concentrations showing similar morphological differentiation inhibition. In this study first the effects on cell viability were determined with a resazurin cytotoxicity assay. Subsequently, differential gene expression responses of selected genes were studied in non cytotoxic concentration ranges of VPA and CBZ.

2. Methods

2.1. Human embryonic stem cell culture

Human embryonic stem cells (hESC) (WA09-DL11, WiCell, Madison, Wisconsin) were cultured in 6-well plates (Corning, NY, Cat#3516) in hESC culture medium (CM), containing: DMEM-F12 (Gibco, Gaithersburg, MD, Cat#31330-038) supplemented with 20% Knock Out Serum Replacement (KOSR) (Gibco, Gaithersburg, MD, Cat#10828), 1 mM L-Glutamine (Gibco, Gaithersburg, Cat#25030-024), 0.5% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070), 1% non-essential amino acids, (Gibco, Gaithersburg, MD, Cat#11140-035), 0.1 mM β-Mercaptoethanol (Sigma–Aldrich, Zwijndrecht, Cat#31350-01) and 0.2 μg/ml fibroblast growth factor-basic(bFGF) (Gibco, Gaithersburg, MD, Cat#13256-029). To maintain pluripotency, the hESCs were cultured on inactivated mouse embryonic fibroblasts. hESC culture medium was refreshed every day.

2.2. Mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) (ATCC, Wesel, Germany CF-1 SCRC-1040) were cultured in T175 culture flasks in MEF culture medium (MM), containing: DMEM (Gibco, Gaithersburg, MD, Cat#11960-044) supplemented with 15% Fetal bovine serum (FBS) Hyclone, Logan, UT, Cat#SH30070.03), 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070), 1% 100 mM Sodium Pyruvate (Gibco, Gaithersburg, MD, Cat#11360-039) and 2 mM L-Glutamine (Gibco, Gaithersburg, MD, Cat#25030-024). When 90% confluent, the cells were incubated with 10 μg/ml Mitomycin C (MMC) (M0503, Sigma–Aldrich, Zwijndrecht, The Netherlands) for 3 h at 37 °C to mitotically inactivate the cells. Subsequently, the MMC solution was removed and the cells were washed with MM followed by Dulbecco's Phosphate-Buffered Salin D-PBS with Ca^{2+} and Mg^{2+} (PBS +/+) (Gibco, Gaithersburg, MD, Cat#14040-174). To detach the cells they were incubated with Trypsin-EDTA (Gibco, Gaithersburg, MD, Cat#25200-056) for 1–2 min. Trypsin was inactivated by adding MM at twice the volume of trypsin, and a single cell suspension was produced by gently pipetting the suspension up and down. After the cells were transferred to 15 ml tubes, they were centrifuged 6 min at 300 rpm at 37 °C. The cells were either stored in liquid nitrogen at $1.2 \cdot 10^6$ cells/ml per vial or directly used as feeder cells at $2 \cdot 10^5$ cells/ well. Inactivated MEFs were seeded into wells and incubated for 24 h in a humidified atmosphere (37 °C 5%CO2) to attach and were used at maximum up to two weeks after seeding.

2.3. hESC culture

hESC were routinely cultured on a layer of inactivated MEFs and passaged between 1 and 3 times per week, depending on growth speed and morphological quality. Since the cell number cannot be controlled, and hESC cells were passaged in fragments, the cell clusters transferred to new inactivated MEF coated dishes differed in size (Fig. 2A). After most of the clusters achieved sufficient size, differentiated clusters and areas were manually removed during the culture period, the optimal clusters were passaged again. Culture medium was refreshed every day. Based on the morphological

quality of the full-grown hESCs clusters, they were passaged either mechanically, by using a dissection needle, or enzymatically with 1 mg/ml Collagenase IV (Gibco, Gaithersburg, MD, Cat#17104-019). For enzymatic passaging, the cells were incubated at 37 °C with 1 mg/ml Collagenase IV for 5–10 min. After the edges of cell clusters curled up microscopically observable, the Collagenase IV solution was discarded and the cells were washed with DMEM-F12 (Gibco, Gaithersburg, MD, Cat#31330-038). Additionally, 2 ml of CM was added per well. The clusters were manually removed by gently scraping with a 5 ml pipet, while simultaneously dispensing the medium. The cell clusters were transferred to a 15 ml tube. The clusters were dispersed into smaller clusters by gently pipetting up and down with a 15 ml pipet. Finally, the cell suspension was subdivided across new wells, containing inactivated MEFs, in a 1:2–1:3 ratio, depending on the amount of cell clusters. The H9 cell batches received from WiCell had passage number 27/28. We could culture them in undifferentiated state up to at least passage 60 without microscopically observed morphological changes. We did not do karyotyping during subculture. The experiments in this manuscript were carried out with passage numbers 36/37, which is ten passages beyond the initial cell batch.

2.4. hESC neural differentiation

Four or five days after the hESC clusters were passaged and cultured on inactivated MEFs, they were enzymatically dissociated by incubating with 1 mg/ml Collagenase IV (Gibco, Gaithersburg, MD, Cat#17104-019) for 10–15 min, until the edges of the hESC cultures curled up and mostly detached from the MEFs. After removal of the Collagenase the cells were washed with DMEM-F12 (Gibco, Gaithersburg, MD, Cat#31330-038) and 2 ml of CM was added to the wells. The cells were collected with a 5 ml pipet from the bottom of the well, while simultaneously dispensing medium. For gene expression analysis the hESC clusters were pooled and subsequently transferred to bacterial dishes (60 × 15 mm, Greiner Cat#628103) containing 3 ml of CM (final volume 5 ml). The cells were incubated for four days in a humidified atmosphere (37 °C 5%CO2). At day 4 the cell aggregates were transferred to a 15 ml tube. After the aggregates sank to the bottom of the tube, the CM was removed and replaced by 2 ml ITS medium; DMEM-F12 (Gibco, Gaithersburg, MD, Cat#31330-038), supplemented with 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070), 1.5 mM L-Glutamine (Gibco, Gaithersburg, MD, Cat#25030-024) and 10% ITS premix (BD Bioscience, Bedford, MA, Cat#354350). Subsequently, the aggregates were cultured for 3 days on Poly-D-Lysine (PDL) (0.1 mg/ml, Sigma–Aldrich, Zwijndrecht, Cat# P6407) and Laminin (0.01 mg/ml Sigma Sigma–Aldrich, Zwijndrecht, Cat# L2020) coated tissue culture dishes 35 × 10 mm (Corning Incorporated, Corning, NY, USA Cat#430165). At day 7 the ITS medium was replaced by Neurobasal medium (Gibco, Gaithersburg, MD, Cat#21103-049), supplemented with N-2 premix (Gibco, Gaithersburg, MD, Cat#17502-048), B27 premix (Gibco, Gaithersburg, MD, Cat#17504-044) and 1% 5000 IU/ml Penicillin/5000 μg/ml Streptomycin (Gibco, Gaithersburg, MD, Cat#15070). After two days the N2/B27 medium was refreshed and cells were cultured for two additional days.

2.5. Immuno staining

Cells attached in culture dishes were washed with cold PBS Ca2+ and Mg2+ free (PBS −/−) (Gibco, Gaithersburg, MD, Cat#14190-094) and fixed with cold 4% paraformaldehyde (Sigma–Aldrich, Zwijndrecht, The Netherlands, Cat#604380) for 10 min at room temperature. The cells were washed twice with cold PBS (−/−), permeabilized in 0.2% Triton X-100 (Sigma–Aldrich, Zwijndrecht, The Netherlands, Cat#93443) for 5 min at 4 °C. After the cells were rinsed twice with PBS (−/−), they were incubated 1 h at 37 °C with blocking buffer: 1% Bovine serum albumin (BSA) (Sigma–Aldrich, Zwijndrecht, The Netherlands, Cat#3808), 0.5% Tween-20 (Sigma–Aldrich, Zwijndrecht, The Netherlands, Cat#93773) in PBS (−/−). The cells were washed with PBS (−/−) and incubated with the primary antibody (AB) overnight at 4 °C. Stem cells were characterized using mouse anti-SSEA-4 (Millipore, Billerica, MA, USA, Cat# FCMAB4304) which binds to the stage-specific embryonic antigen-4, (Thomson et al., 1998), at a 1:3000 dilution within dilution buffer containing 0.5% BSA and 0.5% Tween-20. Neural differentiation was characterized using rabbit anti-βIII-tubulin (Sigma–Aldrich, Zwijndrecht, The Netherlands, Cat#T2200) (Roskams et al., 1998), at a 1:3000 dilution within dilution buffer. After incubation the cells were rinsed with PBS (−/−) and incubated with secondary AB for 1 h at 37 °C. Anti-SSEA4 was incubated with 1:200 diluted Goat anti-mouse IgG γ Chain specific TRITC conjugated polyclonal antibody (Millipore, Billerica, MA, USA, Cat#AP503R). Anti-βIII-tubulin was incubated with 1:200 diluted swine anti-rabbit FITC Dako, Glostrup, Denmark, Cat#F0205). After incubation the cells were washed with PBS (−/−) and nuclei were stained with ProLong Gold antifade reagent with DAPI (Lifetechnologies, Carlsbad, CA, USA Cat#P36934).

2.6. Cytotoxicity

hESC were allowed to adhere for 2 h on a Matrigel coated 96-well plate at 2000 cells/well at humidified atmosphere (37 °C 5%CO2) with addition of 10 μM ROCK inhibitor Millipore, Billerica, MA, USA, Cat# 688000) to the hESC medium. After attachment of the cells to the wells, the cells were exposed to either 0.0033–2.0 mM VPA (Sigma–Aldrich, Zwijndrecht, The Netherlands, CAS#1069-66-5) or 0.0033–2.0 mM CBZ Sigma–Aldrich, Zwijndrecht, The Netherlands, CAS#298-46-4) (Solved in 0.25% DMSO) in hESC medium for 5 days, at 6 technical replicates per concentration. After 3 days the medium was refreshed, containing the same test compound and concentration. At day 5 the cell viability was determined using Cell-titer blue viability assay (Promega, Leiden, The Netherlands, Cat#G8081), in which the cells were incubated with resazurin for 4 h at 37 °C. Viable cells were able to convert resazurin (a redox dye) into a fluorescent product (resofurin) which was measured with FLUOstar spectrofluorometer (FLUOstar Optima, BMG Labtech, de Meeren, The Netherlands) at 544 nm (excitation) and 590 nm (emission). Via concentration response curves using a log-logistic model, generated with Proast software (Slob, 2002), the compound concentration inducing 50% inhibition of cell viability (IC50), was calculated.

2.7. Gene expression analysis

To study the gene expression over time during the differentiation period, samples were collected at day 0 ($n = 6$), 1 ($n = 6$), 4 ($n = 2$), 7 ($n = 6$), 9 ($n = 2$) and 11 ($n = 2$). All cultures originated from the same pool of cells, collected from a large group of undifferentiated hESC cultures. Per sample 500 ng RNA was reversed transcribed into complementary DNA (cDNA) by using the High capacity cDNA reverse transcriptase kit (Applied Biosystems Inc., Foster City, CA, USA, Cat# 4387406). Gene expression was measured by Real-time PCR (RT-PCR) using TaqMan fast universal PCR Master mix (Applied Biosystems Inc., Foster City, CA, USA, Cat#4366073) and specific TaqMan Gene Expression Assays (Applied Biosystems Inc., Foster City, CA, USA). Genes both associated with self-renewal and maintenance of pluripotency of stem cells POU domain class 5 transcription factor 1 (POU5F1) (Applied Biosystems Cat#Hs00999632_g1) and (Nanog) (Applied Biosystems Cat#Hs02387400_g1) as well as genes involved in neural

differentiation and associated with neural cells βIII-tubulin (TUBB) (Applied Biosystems Cat#Hs00964962_g1) Microtubule-associated protein 2 (MAP2) (Applied Biosystems Cat#Hs00258900_m1) Neurogin1 (Applied Biosystems Cat#Hs01029249_m1) Microtubule-associated protein tau (MAPT) (Applied Biosystems Cat#Hs00902194_m1) and Reelin (Reln) (Applied Biosystems Cat#Hs01022646_m1) were validated to be used with Housekeeping genes hypoxanthine guanine phosphoribosyltransferase (HPRT) (Applied Biosystems Cat#Hs02800695_m1) and Glucuronidase beta (GUSb) (Applied Biosystems Cat#Hs00939627_m1) to calculate ΔCt values. Gene expression levels were corrected for the average Ct values of the 2 house keeping genes and the control (ΔΔCt) ABI Prism 7700 Sequence (1997).

2.8. RNA extraction

Cells ready for RNA extraction were stored at −20 °C in RNA protect (Qiagen Benelux, Venlo, The Netherlands, Cat# 76526). RNA was extracted using the Qiacube (Qiagen) and a Qiagen RNA Mini-extraction kit (Qiagen, Cat#74104) including a DNAse treatment step (Qiagen Cat# 79254) following the manufacturer's protocol. The extracted RNA was eluted in RNase free water and stored at −80 °C. RNA concentrations were measured using the Nanodrop (Thermo Scientific).

2.9. Compound mediated effect on differentiation

Cells were exposed to 0.033 ($n = 2$), 0.1 ($n = 2$), 0.33 mM ($n = 4$) and 1.0 mM ($n = 4$) VPA (Sigma–Aldrich, Zwijndrecht, The Netherlands, CAS#1069-66-5) or 0.01 mM ($n = 2$), 0.033 ($n = 2$), 0.1 mM ($n = 4$) and 0.33 ($n = 3$) CBZ (Sigma–Aldrich, Zwijndrecht, The Netherlands, CAS#298-46-4) starting at initiation of differentiation (day 0) and continued until day 7.

Since all genes studied were significantly regulated in control cultures after 7 days, the cells were exposed from day 0 until day 7 of the differentiation period and RNA of exposed and non-exposed samples was extracted at culture day 7.

2.10. Data analysis

Gene expression was studied using Graphpad Prism (version 6). Statistics were calculated by using analysis of Graphpad Prism in which the mean of each group was compared with the mean of the solvent control with post-hoc *t*-test (Dunnett's multiple comparison test, with a 95% confidence interval). Significance of concentration response was calculated with a Post-hoc *t*-test for linear trend. Indication of a *P*-value < 0.0001: ****, 0.0001–0.001: ***, 0.001–0.01: ** and 0.01–0.05: *

3. Results

3.1. hESC cell culture

hESC were routinely cultured and passaged 1–3 times per week. As the hESC cell cultures were passaged in fragments, the resulting cell clusters adhering to new inactive MEF coated dishes differed in size (Fig. 1A and B). The cultures were observed daily and examined morphologically both by light microscopy and by fluorescent immuno-staining with anti-SSEA4. Within the cultures of undifferentiated hESC, usually some spontaneously differentiated cells were present, expressing βIII-tubulin observed after immunostaining (Fig. 1C).The extent of βIII-tubulin expressing cells was very low and stable with increasing passage number. Quality of the cells was monitored using immuno staining for SSEA-4 and βIII-tubulin and by RT-PCR analysis for all genes employed in this study.

3.2. hESC differentiation

Cells were cultured in CM for 4 days in which cell aggregates were formed (Fig. 2A and B). At day 4 the cells were transferred to PDL and Laminin coated dished in which the aggregates attached to the bottom of the dishes (Fig. 2C). The cells were cultured in ITS containing medium until day 7 during which cells migrated out of the aggregates and dendritic structures became visible (Fig. 2C). At day 7 the ITS culture medium was replaced by Neurobasal medium containing N2 en B27. After 11 days the cultures consisted of cells representing neural cell morphology with relatively few cells expressing stem cell marker SSEA4, as demonstrated with immunological staining with anti-SSEA4 and anti-βIII-tubulin (Fig. 2D–G).

3.3. RT-PCR analysis of hESC renewal and differentiation

The expression of genes involved in stem cell renewal and maintenance of pluripotency, as well as marker genes for neural differentiation were studied using RT-PCR analysis (Fig. 3). After 4 days the gene expression of both stem cell markers Pou5F1 and Nanog had significantly decreased, with a continuing decrease in expression until day 11. The genes involved in neurogenesis showed a significant up-regulation over time. Already 24 h after initiation of neural differentiation βIII-tubulin, Map2 and Neurog1 were significantly up-regulated. After 7 days, all genes studied involved in neurogenesis were significantly upregulated.

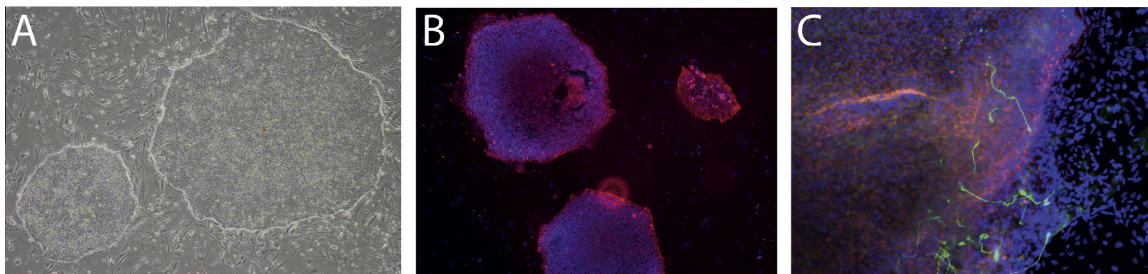

Fig. 1. Culture of pluripotent human embryonic stem cells. (A) H9 hESC were cultured on mitotically inactivated MEFs (magnification: 40×). (B) SSEA4 immunostaining of an undifferentiated hESC cluster (red). Cell nuclei were stained with DAPI (blue) (magnification: 40×). (C) βIII-tubulin immunostaining of some spontaneously differentiated neural cells in hESC clusters (green). Undifferentiated hESC clusters were stained with anti-SSEA4 (red). Cell nuclei were stained with DAPI (blue) (magnification 100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

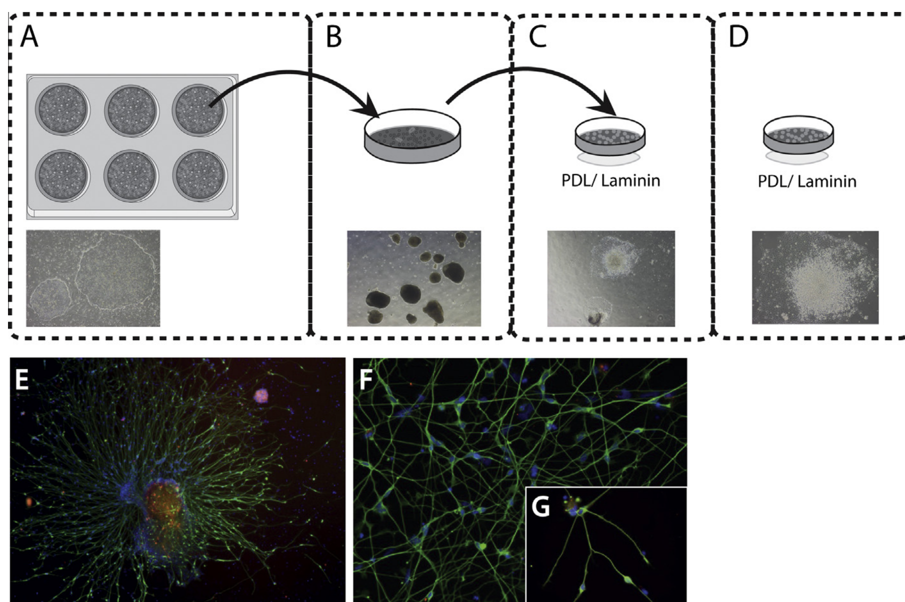

Fig. 2. Neural differentiation method. (A) After enzymatic dissociation, hESC clusters were transferred to culture dishes containing CM and cultured 4 days to form cell aggregates. (B) At day 4 the aggregates were transferred to PDL/laminin coated dished and cultured in ITS medium for 3 days. The aggregates attached to the dishes and cells started to migrate out of the aggregates. (C) At day 7 the ITS medium was replaced by N2 medium and cultures were continued for 4 days. Neural structures became visible. (D–G) At day 11 there were very few SSEA4 positive cells present (red), and a network of cells, expressing βIII-tubulin (green) had formed. Cell nuclei were stained with Dapi (blue). (Magnification E: 40×, F: 100×, G: 100×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

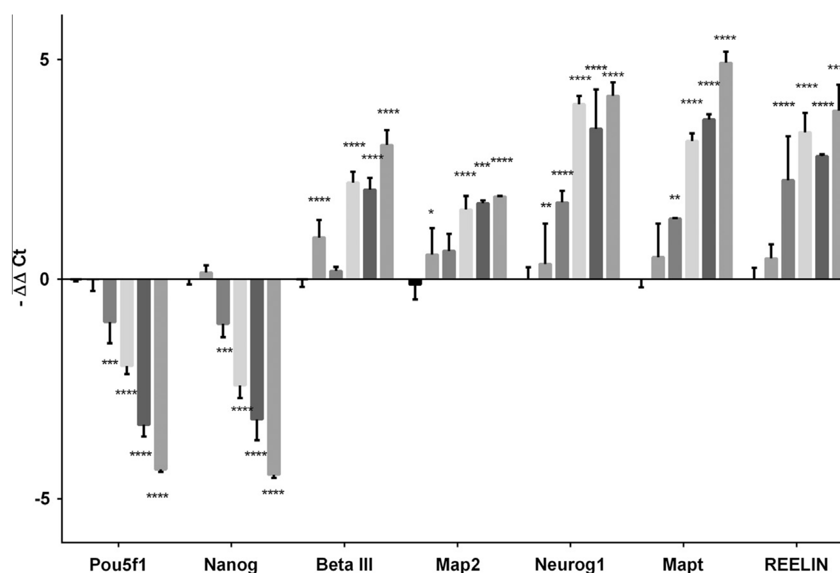

Fig. 3. Relative gene expression of stem cell- and neural development related genes during differentiation. The expression of stem cell related genes, Pou5F1 and Nanog, together with genes expressed in neural development, βIII-tubulin, Map2, Neurogin1, Mapt and Reelin, were measured at day 0 (■), 1 (■), 4 (■), 7 (■), 9 (■) and 11(■).

3.4. Effects of compounds on hESC viability

Concentration responses in the hESC cytotoxicity assay resulted in IC50 values of 1.71 mM of VPA (Fig. 4A) and 0.436 mM of CBZ (Fig. 4B).

3.5. Effect of compounds on gene expression in differentiating hESC

The effects of VPA and CBZ on differentiation were tested by gene expression at concentrations below the IC50. Both for VPA and CBZ a concentration related effect on the expression of most of the genes studied was observed at day 7 of differentiation. (Figs. 5 and 6).

Exposure to VPA caused a significant concentration dependent regulation of gene expression. There was a decrease in expression of the stem cell related genes Pou5F1 and Nanog. The neural development related genes βIII-tubulin, Neurog1 and Reelin, were all significantly down regulated in a concentration dependent response, whereas both Map2 and Mapt showed a significant concentration dependent increased expression compared to the control. CBZ did not cause a significant concentration response of the stem cell related genes Pou5F1 and Nanog. Of the neural developmental involved genes, Map2 gene expression did not respond

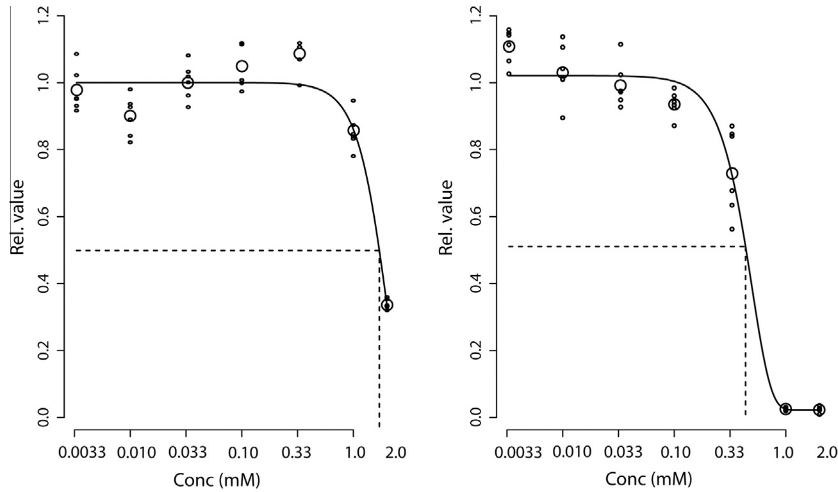

Fig. 4. Cytotoxicity concentration–response curves in undifferentiated hESC culture of VPA (IC50 1.71 mM) (A) and CBZ (IC50 0.436 mM) (B).

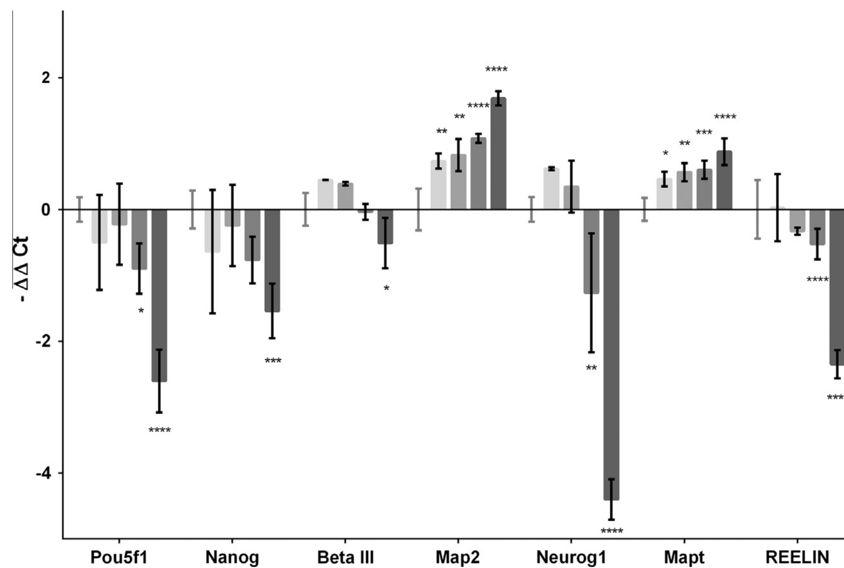

Fig. 5. The effect of VPA exposure on gene expression in hESTn. Differentiating cells were exposed from day 0 to 7 to increasing concentrations of VPA; Solvent control (0.25% DMSO) (), 0.033 mM (), 0.1 mM (), 0.33 mM () and 1.0 m (). The effect on stem cell related genes, Pou5F1 and Nanog, and on genes involved in neural development, βIII-tubulin, Map2, Neurogin1, Mapt and Reelin, was measured at day 7 and compared to the average ΔΔCt of gene expression in unexposed differentiating cells at day 7.

significantly to CBZ exposure. However, βIII-tubulin, Neurog1, Mapt and Reelin all had a significant concentration-response and showed a down regulation in response to an increasing concentration of CBZ.

4. Discussion

Defects of the neural tube are among the most common malformations in mammalian species (Gilbert-Barness, 2010). Mouse ESC have proven a relatively easy starting point for neuro developmental toxicity assay design. Theunissen et al. (2010) however, in view of interspecies differences hESC could provide improved predictability of the assay for developmental toxicity in man. During the last decade several *in vitro* developmental toxicity assays based on hESC have been developed, (Buzanska et al., 2009; Krug et al., 2013; Adler et al., 2008a; Hoelting et al., 2013; Meganathan et al., 2012; Colleoni et al., 2012; Adler et al., 2008b). They have been based on various cell types, such as stem cells from umbilical cord blood (Buzanska et al., 2009), induced pluripotent stem cells (IPS) Ebert et al. (2013), neural progenitor cells (Axell et al., 2009; Dhara et al., 2008) and hESC (Iacovitti et al., 2007). Some methods made use of primary cultures of neural cell rosettes (Colleoni et al., 2011) or neurospheres (Hoelting et al., 2013). These tests are usually complex in terms of culture conditions, and have a long duration, up to several weeks, which provide practical disadvantages as compared to mESTn. Additionally, they often focus on a single developmental process, such as cell proliferation, embryoid body formation or neurite outgrowth (Krug et al., 2013; Colleoni et al., 2011). We developed a relatively short duration- and simple culture procedure encompassing different developmental processes, i.e. cell proliferation, aggregate formation, neural cell differentiation, and neurite outgrowth. Our method presented here is based on a commercially available human embryonic stem cell line H9, employed extensively in embryonic cell differentiation research (Loser et al., 2010). Furthermore, this hESC differentiation assay is comparable in duration to the mouse embryonic stem cell

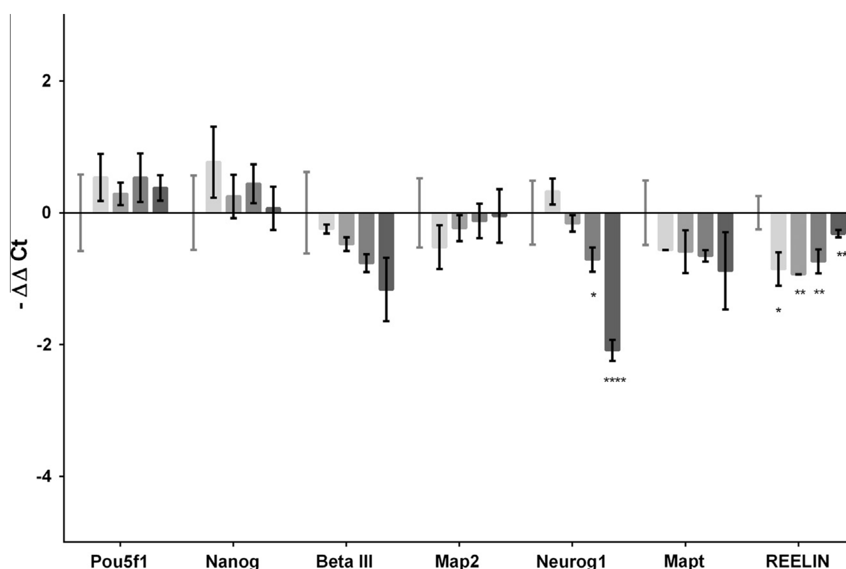

Fig. 6. The effect of CBZ exposure on gene expression in hESTn. Differentiating cells were exposed from day 0 to 7 to increasing concentrations of CBZ; Solvent control (medium) ( ), 0.01 mM ( ), 0.033 mM ( ), 0.1 mM ( ) and 0.33 mM ( ). The effect on stem cell related genes, Pou5F1 and Nanog, together with genes involved in neural development, βIII-tubulin, Map2, Neurogin1, Mapt and Reelin, was measured at day 7 and compared to the average ΔΔCt of gene expression in unexposed differentiating cells at day 7.

neural differentiation assay described by Theunissen et al. (2010). This facilitates comparative studies of compound effects among EST assays based human versus murine stem cells.

Loss of stemness and gain of neural cell characteristics in our assay was studied with a set of 7 marker genes. Both Nanog and Pou5F1 are transcription factors involved in pathways controlling pluripotency and self-renewal by molecular reprogramming (Loh et al., 2006; Silva et al., 2009). Neural differentiation was studied with βIII-tubulin, a microtubule element exclusively expressed within neurons (Kintner, 2002). Microtubule associated proteins (MAPs) mediate interaction between neurons and microtubule elements. MAP2 is a high molecular weight MAP, and MAPtau (MAPt) has a low molecular weight (Alberts et al., 1994). Neurogin1, a basic-helix-loop-helix (bHLH) transcription factor, plays a role in neural tube patterning and folding after neuro-ectoderm has formed (Velkey and O'Shea, 2013). Furthermore, Neurogin1 slows proliferation and promotes neural differentiation and neuronal subtype specification of glutaminergic and GABAergic linages (Velkey and O'Shea, 2013). Reelin plays a role in neural GABAergic Purkinje cell migration from the proliferative areas toward the pial surface of the cerebral cortex during corticogenesis. (Miyata et al., 2010; Jossin, 2004; Nomura et al., 2008).

In our hESTn, whereas both stem cell markers Nanog and Pou5F1 showed a decrease over time, all neural differentiation related genes studied showed a significant increase in expression, demonstrating neural differentiation. Already 24 h after initiation of differentiation, significant increases were observed in the expression of neural markers βIII-tubulin, Map2 and Neurogin1, in contrast to no significant changes in expression of the stem cell related genes. This suggests continued stem cell proliferation at these early stages in the differentiation protocol, in the presence of increasing differentiation into neural cells. Virtual absence of stem cells at day 11 in the assay was indicated by very low numbers of cells expressing SSEA-4. In the mouse ESTn (Theunissen et al., 2010), which is comparable in terms of the time course of differentiation, there was little to no increase in the expression of neural genes occurring during the first three days of the mESC neural differentiation method. This dissimilarity may partly be due to the differences in initial cell aggregate formation between methods. Moreover, in contrast to mouse ES cell cultures, 'undifferentiated' human ES cell cultures were shown to systematically contain some cells expressing βIII-tubulin, albeit very few, which may explain the faster progression of neural differentiation progression in the human versus the mouse based ESTn assays.

VPA and CBZ are anticonvulsant pharmaceuticals both of which exert their pharmacological effect via increasing GABA- and Glutamatergic neurotransmitter levels (Nanau and Neuman, 2013; Lloyd, 2013). Furthermore, VPA has neuro-protective effects by stimulating neural differentiation and neurite outgrowth, caused by the inhibition of the release of pro-inflammatory factors, inhibition of the GSK3/PI3-kinase/Akt/NFκβ pathway, the release of neurotrophic factors, like GDNF and BDNF and the inhibition of histone deacetylase enzymes (HDAC) Brunn et al. (2014); Almutawaa et al. (2014) with a beneficial effect in neurodegenerative conditions (Brunn et al., 2014; Biermann et al., 2010). However, HDAC inhibition and consequent chromatin remodeling during early development is suggested to cause (neuro) developmental abnormalities (Lloyd, 2013; Brunn et al., 2014). Therefore, these anticonvulsant drugs have been associated both with neurotoxic as well as neuroprotective characteristics. In our hESTn assay, in therapeutic concentration ranges both compounds affected the expression of genes involved in pluripotency and/or neuro-development, in a compound and concentration specific response. VPA clearly showed a differentiation stimulus, evidenced by a decrease of stem cell related genes and an increase in the expression of βIII-tubulin and Neurogin1, Map2 and MAPt. This is reminiscent of the neuroprotective and remodeling effect and the induction of neural outgrowth caused by VPA exposure (Dou et al., 2003; Yu et al., 2009). However, with increasing concentration the expression of βIII-tubulin and Neurogin1 was inhibited, together with an inhibition of Reelin expression across all concentrations. This supports the assumption that the nature of the VPA effect is concentration dependent, with a neuroprotective effect at lower exposures and a neurotoxic effect at higher exposures (Alsdorf and Wyszynski, 2005). Therefore, therapeutic concentrations which are beneficial for the pregnant woman may in parallel cause developmental toxicity, since VPA is known to accumulate within the embryo (Lloyd, 2013; Alsdorf and Wyszynski, 2005; Nau and Scott, 1987). These effects of VPA in hESTn were qualitatively similar to earlier findings in mESTn in a whole genome array study after 24 h exposure

in the same concentration range (Theunissen et al., 2012a,b). A VPA-induced decreased expression of βIII-tubulin, Pou5f1 and Neurogin1 was seen in both the mESTn and hESTn. In parallel, an increased expression of Mapt and Map2 was observed in both models upon VPA exposure.

CBZ affected gene expression to a lesser extent compared to VPA. This difference in the nature of the responses is in agreement with the effects found *in vivo*, in which CBZ also has been demonstrated to be less teratogenic (Jentink et al., 2010; Ornoy, 2006). CBZ exposure did not show effects on the expression of the stemness related genes. However, βIII-tubulin, Neurogin1, MAPt and Reelin were all down regulated, with a significant concentration dependent trend, suggesting an inhibiting effect on neural cell differentiation. The mechanism of action of CBZ has not been revealed as extensively as for VPA. However it is known that CBZ also is an inhibitor of HDAC, which has been linked to its teratogenic effect (Hill et al., 2010; Beutler et al., 2005). No significant effects on the selected genes of this study were observed in the mESTn, measured after 24 h exposure to CBZ (Theunissen et al., 2012a). However, a direct comparison of quantitative responses is precluded by necessary differences in culture conditions and assay design.

Taken together, we accomplished a rapid, standardized hESC neural differentiation assay for neurodevelopmental toxicity. Unexposed differentiated cell cultures had lost their pluripotency markers and consisted largely of neural cells expressing βIII-tubulin, as confirmed with immuno-staining. Given that VPA and CBZ caused unique compound specific changes in gene expression in the assay, we demonstrated the responsiveness of the model to neurodevelopmental toxicants. This neurodevelopmental toxicity assay will be further studied for application as a rapid first tier screening for the detection of potential neurodevelopmental toxicants.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

References

ABI Prism 7700 Sequence Detection System. User Bulletin #2 The Perkin-Elmer Corporation, 1997. <http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf>.

Adler, S. et al., 2008a. Testing potential developmental toxicants with a cytotoxicity assay based on human embryonic stem cells. Altern. Lab Anim. 36 (2), 129–140.

Adler, S. et al., 2008b. First steps in establishing a developmental toxicity test method based on human embryonic stem cells. Toxicol. In Vitro 22 (1), 200–211.

Alberts, B., Bray, D., Lewis, J., et al., 1994. Molecular Biology of the Cell, third ed. Garland Science, New York, Microtubules. http://www.ncbi.nlm.nih.gov/books/NBK28419/.

Almutawaa, W., et al., 2014. Induction of neurotrophic and differentiation factors in neural stem cells by valproic acid. Basic Clin. Pharmacol. Toxicol.

Alsdorf, R., Wyszynski, D.F., 2005. Teratogenicity of sodium valproate. Exp. Opin. Drug Saf. 4 (2), 345–353.

Axell, M.Z., Zlateva, S., Curtis, M., 2009. A method for rapid derivation and propagation of neural progenitors from human embryonic stem cells. J. Neurosci. Methods 184 (2), 275–284.

Beutler, A.S. et al., 2005. Carbamazepine is an inhibitor of histone deacetylases. Life Sci. 76 (26), 3107–3115.

Biermann, J. et al., 2010. Valproic acid-mediated neuroprotection and regeneration in injured retinal ganglion cells. Invest. Ophthalmol. Vis. Sci. 51 (1), 526–534.

Brunn, J. et al., 2014. Valproic acid in normal therapeutic concentration has no neuroprotective or differentiation influencing effects on long term expanded murine neural stem cells. Epilepsy Res. 108 (4), 623–633.

Buzanska, L. et al., 2009. A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. Stem Cells 27 (10), 2591–2601.

Colleoni, S. et al., 2011. Development of a neural teratogenicity test based on human embryonic stem cells: response to retinoic acid exposure. Toxicol. Sci. 124 (2), 370–377.

Colleoni, S. et al., 2012. Characterisation of a neural teratogenicity assay based on human ESCs differentiation following exposure to valproic acid. Curr. Med. Chem. 19 (35), 6065–6071.

de Jong, E., van Beek, L., Piersma, A.H., 2012. Osteoblast differentiation of murine embryonic stem cells as a model to study the embryotoxic effect of compounds. Toxicol. In Vitro 26 (6), 970–978.

van der Jagt, K., et al., Alternative approaches can reduce the use of test animals under reach. European Commission Report, 2004. EUR 21405 EN.

Dhara, S.K. et al., 2008. Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures. Differentiation 76 (5), 454–464.

Dou, H. et al., 2003. Neuroprotective activities of sodium valproate in a murine model of human immunodeficiency virus-1 encephalitis. J. Neurosci. 23 (27), 9162–9170.

Ebert, A.D. et al., 2013. EZ spheres: a stable and expandable culture system for the generation of pre-rosette multipotent stem cells from human ESCs and iPSCs. Stem Cell Res. 10 (3), 417–427.

Fathi, A. et al., 2011. Comprehensive gene expression analysis of human embryonic stem cells during differentiation into neural cells. PLoS One 6 (7), e22856.

Gilbert-Barness, E., 2010. Teratogenic causes of malformations. Ann. Clin. Lab Sci. 40 (2), 99–114.

Gurvich, N., Klein, P.S., 2002. Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. Pharmacol. Ther. 96 (1), 45–66.

Heuer, J. et al., 1993. Development of an in vitro embryotoxicity test using murine embryonic stem cell cultures. Toxicol. In Vitro 7 (4), 551–556.

Hill, D.S. et al., 2010. Teratogenic effects of antiepileptic drugs. Exp. Rev. Neurother. 10 (6), 943–959.

Hoelting, L. et al., 2013. A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles. Arch. Toxicol. 87 (4), 721–733.

Iacovitti, L. et al., 2007. A protocol for the differentiation of human embryonic stem cells into dopaminergic neurons using only chemically defined human additives: studies in vitro and in vivo. Brain Res. 1127 (1), 19–25.

Jentink, J. et al., 2010. Intrauterine exposure to carbamazepine and specific congenital malformations: systematic review and case-control study. BMJ 341, c6581.

Jones, K.L. et al., 1989. Pattern of malformations in the children of women treated with carbamazepine during pregnancy. N. Engl. J. Med. 320 (25), 1661–1666.

Jossin, Y., 2004. Neuronal migration and the role of reelin during early development of the cerebral cortex. Mol. Neurobiol. 30 (3), 225–251.

Kintner, C., 2002. Neurogenesis in embryos and in adult neural stem cells. J. Neurosci. 22 (3), 639–643.

Kou, W. et al., 2011. In vitro and in vivo evaluation of novel immediate release carbamazepine tablets: complexation with hydroxypropyl-beta-cyclodextrin in the presence of HPMC. Int. J. Pharm. 409 (1–2), 75–80.

Krug, A.K. et al., 2013. Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach. Arch. Toxicol. 87 (1), 123–143.

Liu, W. et al., 2013. Stem cell models for drug discovery and toxicology studies. J. Biochem. Mol. Toxicol. 27 (1), 17–27.

Liu, C. et al., 2013. Neural differentiation of human embryonic stem cells as an in vitro tool for the study of the expression patterns of the neuronal cytoskeleton during neurogenesis. Biochem. Biophys. Res. Commun. 439 (1), 154–159.

Lloyd, K.A., 2013. A scientific review: mechanisms of valproate-mediated teratogenesis. Biosci. Horizons 6, 1–10.

Loh, Y.H. et al., 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. 38 (4), 431–440.

Loser, P. et al., 2010. Human embryonic stem cell lines and their use in international research. Stem Cells 28 (2), 240–246.

Meganathan, K. et al., 2012. Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of human embryonic stem cells. PLoS One 7 (8), e44228.

Miyata, T. et al., 2010. Migration, early axonogenesis, and Reelin-dependent layer-forming behavior of early/posterior-born Purkinje cells in the developing mouse lateral cerebellum. Neural Dev. 5, 23.

Mori, H., Hara, M., 2013. Cultured stem cells as tools for toxicological assays. J. Biosci. Bioeng. 116 (6), 647–652.

Nanau, R.M., Neuman, M.G., 2013. Adverse drug reactions induced by valproic acid. Clin. Biochem. 46 (15), 1323–1338.

Nau, H., Scott, W.J., 1987. Teratogenicity of valproic acid and related substances in the mouse: drug accumulation and pHi in the embryo during organogenesis and structure-activity considerations. Arch. Toxicol. Suppl. 11, 128–139.

Nomura, T. et al., 2008. Patterns of neurogenesis and amplitude of Reelin expression are essential for making a mammalian-type cortex. PLoS One 3 (1), e1454.

Ornoy, A., 2006. Neuroteratogens in man: an overview with special emphasis on the teratogenicity of antiepileptic drugs in pregnancy. Reprod. Toxicol. 22 (2), 214–226.

Ornoy, A., Cohen, E., 1996. Outcome of children born to epileptic mothers treated with carbamazepine during pregnancy. Arch. Dis. Child 75 (6), 517–520.

Robert, E., Guibaud, P., 1982. Maternal valproic acid and congenital neural tube defects. Lancet 2 (8304), 937.

Rosa, F.W., 1991. Spina bifida in infants of women treated with carbamazepine during pregnancy. N. Engl. J. Med. 324 (10), 674–677.

Roskams, A.J., Cai, X., Ronnett, G.V., 1998. Expression of neuron-specific beta-III tubulin during olfactory neurogenesis in the embryonic and adult rat. Neuroscience 83 (1), 191–200.

Scholz, G. et al., 1999. Prevalidation of the embryonic stem cell test (EST)-a new in vitro embryotoxicity test. Toxicol. In Vitro 13 (4–5), 675–681.

Schulz, T.C. et al., 2003. Directed neuronal differentiation of human embryonic stem cells. BMC Neurosci. 4, 27.

Silva, J. et al., 2009. Nanog is the gateway to the pluripotent ground state. Cell 138 (4), 722–737.

Slob, W., 2002. Dose–response modeling of continuous endpoints. Toxicol. Sci. 66 (2), 298–312.

Spielmann, H., 2009. The way forward in reproductive/developmental toxicity testing. Altern. Lab Anim. 37 (6), 641–656.

Talens-Visconti, R. et al., 2011. Neural differentiation from human embryonic stem cells as a tool to study early brain development and the neuroteratogenic effects of ethanol. Stem Cells Dev. 20 (2), 327–339.

Theunissen, P.T. et al., 2010. An abbreviated protocol for multilineage neural differentiation of murine embryonic stem cells and its perturbation by methyl mercury. Reprod. Toxicol. 29 (4), 383–392.

Theunissen, P.T. et al., 2012a. Compound-specific effects of diverse neurodevelopmental toxicants on global gene expression in the neural embryonic stem cell test (ESTn). Toxicol. Appl. Pharmacol. 262 (3), 330–340.

Theunissen, P.T. et al., 2012b. Transcriptomic concentration-response evaluation of valproic acid, cyproconazole, and hexaconazole in the neural embryonic stem cell test (ESTn). Toxicol. Sci. 125 (2), 430–438.

Thomson, J.A. et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282 (5391), 1145–1147.

van Dartel, D.A., Piersma, A.H., 2011. The embryonic stem cell test combined with toxicogenomics as an alternative testing model for the assessment of developmental toxicity. Reprod. Toxicol. 32 (2), 235–244.

van Dartel, D.A. et al., 2009. Early gene expression changes during embryonic stem cell differentiation into cardiomyocytes and their modulation by monobutyl phthalate. Reprod. Toxicol. 27 (2), 93–102.

van Dartel, D.A. et al., 2010. Transcriptomics-based identification of developmental toxicants through their interference with cardiomyocyte differentiation of embryonic stem cells. Toxicol. Appl. Pharmacol. 243 (3), 420–428.

van Dartel, D.A. et al., 2011a. Evaluation of developmental toxicant identification using gene expression profiling in embryonic stem cell differentiation cultures. Toxicol. Sci. 119 (1), 126–134.

van Dartel, D.A. et al., 2011b. Discriminating classes of developmental toxicants using gene expression profiling in the embryonic stem cell test. Toxicol. Lett. 201 (2), 143–151.

Velkey, J.M., O'Shea, K.S., 2013. Expression of Neurogenin 1 in mouse embryonic stem cells directs the differentiation of neuronal precursors and identifies unique patterns of down-stream gene expression. Dev. Dyn. 242 (3), 230–253.

Yu, I.T. et al., 2009. Valproic acid promotes neuronal differentiation by induction of proneural factors in association with H4 acetylation. Neuropharmacology 56 (2), 473–480.