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Comparative Neurotoxicity Screening in Human iPSC-derived Neural Stem Cells, Neurons and Astrocytes

Ying Pei[1], **Jun Peng**[2], **Mamta Behl**[3], **Nisha S Sipes**[3], **Keith R Shockley**[3], **Mahendra S Rao**[4], **Raymond R Tice**[3], and **Xianmin Zeng**[1,2]

[1]XCell Science Inc., Novato, CA, USA

[2]Buck Institute for Research on Aging, Novato, CA, USA

[3]National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27713

[4]NxCell Science, Novato, CA

Abstract

Induced pluripotent stem cells (iPSC) and their differentiated derivatives offer a unique source of human primary cells for toxicity screens. Here, we report on the comparative cytotoxicity of 80 compounds (neurotoxicants, developmental neurotoxicants, environmental compounds) in iPSC as well as isogenic iPSC-derived neural stem cells (NSC), neurons, and astrocytes. All compounds were tested over a 24-hour period at 10 and 100 μM, in duplicate, with cytotoxicity measured using the MTT assay. Of the 80 compounds tested, 50 induced significant cytotoxicity in at least one cell type; per cell type, 32, 38, 46, and 41 induced significant cytotoxicity in iPSC, NSC, neurons, and astrocytes, respectively. Four compounds (valinomycin, 3,3′,5,5′-tetrabromobisphenol, deltamethrin, triphenyl phosphate) were cytotoxic in all four cell types. Retesting these compounds at 1, 10, and 100 μM using the same exposure protocol yielded consistent results as compared with the primary screen. Using rotenone, we extended the testing to seven additional iPSC lines of both genders; no substantial difference in the extent of cytotoxicity was detected among the cell lines. Finally, the cytotoxicity assay was simplified by measuring luciferase activity using lineage-specific luciferase reporter iPSC lines which were generated from the parental iPSC line.

Introduction

The human brain is enormously complex and undergoes dramatic changes in cell number, overall size, and morphology during development. The complex pattern of development is carefully orchestrated with timed morphogenetic movement, stage specific regionalization, and cell lineage segregation (Dobbing and Sands, 1973; Ourednik et al., 2001). While other developing organs exhibit a similar pattern, the sheer number of neurons, the complexity of the wiring, and the disproportionately large number of genes that are expressed in the brain

Correspondence: Xianmin Zeng, Ph.D. XCell Science Inc, Novato, CA 94945, USA; Tel: 415-209-2211; xzeng@xcellscience.com.

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render its development potentially more susceptible to environmental influences. However, of the more than 80,000 compounds in commerce, only 11 have been identified as human developmental neurotoxicants while more might remain undiscovered (Grandjean and Landrigan, 2014).

Despite the fact that rodent-based developmental neurotoxicology models have relatively low sensitivity, low throughput, and high cost, they have been the primary approach for detecting potential human neurotoxicants. However, given the enormous difference in size and complexity of the human brain as compared to the brain of rodents, many of the developmental pathways are different or are regulated with additional factors (Clancy et al., 2007; Deacon, 1997; Van Dam and De Deyn, 2006). For example, the sets of genes that control forebrain expansion and regulate human cell fate are largely absent in rodents. Also, fibroblast growth factor (FGF) has different effects on myelination in humans and rodents (Hu et al., 2009), and compounds that are toxic to rodent cells may have no effect on human cells or vice versa (Malik et al., 2014; Xia et al., 2008).

In response to increased concerns about neurotoxicity induced in humans by exposure to chemicals during development, the scientific community is developing alternatives that will reduce the use of traditional laboratory animals while addressing the demand for increased and more relevant testing. In addition, more than 30,000 chemicals without adequate toxicological information are estimated to be in use in the United States and Europe (Schmidt, 2009), and the task of testing thousands of chemicals systematically with classical animal tests exceeds our present capabilities. In 2008, in response to the US National Academy of Sciences' report on "Toxicity Testing in the 21st Century" (NAS, 2007), a collaboration was established between the National Institute of Environmental Health Sciences (NIEHS)/National Toxicology Program (NTP), the U.S. Environmental Protection Agency's (EPA) National Center for Computational Toxicology (NCCT), and the National Human Genome Research Institute (NHGRI)/National Institutes of Health (NIH) Chemical Genomics Center (NCGC) (Collins et al., 2008). In mid-2010, the U.S. Food and Drug Administration (FDA) joined the collaboration, known informally as Tox21. The objective of this partnership is to shift the assessment of chemical hazards from traditional experimental animal toxicology studies to one based on target-specific, mechanism-based, biological observations largely obtained using *in vitro* assays, with the ultimate aim of improving risk assessment for humans and the environment. Additionally, the new European legislation on chemicals – Registration, Evaluation, Authorisation and Restriction of Chemicals (ReACH) – explicitly mentions the possibility of using both experimental (*in vitro*) and non-testing (structure-activity relationships, read-across, categories) alternative methods (ReACH, 2015).

The recent advance in pluripotent stem cell (PSC)-based technology and the ability to generate truly large numbers of allelically diverse cells and use uniform methods to differentiate them into all of the major types of cells offer a potential new tool for improved understanding of chemically-induced adverse reactions. This is especially useful for developmental neurotoxicity, because neural cells differentiate early during development and this process is relatively easy to recapitulate *in vitro* via rosette formation and isolation of neural stem cells (NSC), which can subsequently be differentiated into neurons and glia.

Several groups including our own have developed protocols to differentiate NSC, neurons, astrocytes, and oligodendrocytes from PSCs (Liu et al., 2013; Shaltouki et al., 2013; Swistowski et al., 2009; Swistowski et al., 2010). In addition, we and others have developed lineage specific markers and reporter lines which facilitate high content screening (Efthymiou et al., 2014) to allow us to obtain much more information from a single assay. We have utilized these tools to perform high throughput screens at different stages of development using purified cell populations (Han, 2009; Peng et al., 2013).

Here, we present the results obtained from testing a 80-compound library comprised of drugs (e.g., valproic acid) and pesticides (e.g., aldicarb, rotenone) with known neurotoxic potential as well as environmental compounds with unknown neurotoxic potential (e.g., flame retardants, polycylic aromatic hydrocarbons [PAHs]) for their cytotoxic effect on isogenic cells at four stages of neural differentiation (iPSC, NSC, neuron, astrocyte) using the MTT assay. This assay measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as a measure of cell viability (Berridge et al., 2005; Morgan, 1998). When tested at 10 and 100 μM, 32 (40%) to 46 (58%) of the compounds induced significant cytotoxicity in the four cell types, with cell-type specificity. The results were confirmed by the retesting of four selected compounds that were cytotoxic to all four cell types, and the testing was extended using rotenone to additional iPSC lines of both genders. Finally, we show that the cytotoxicity assay can be simplified by measuring luciferase activity using lineage-specific luciferase reporter iPSC lines that we generated from the parent iPSC line.

Materials and methods

80-compound library

We evaluated cytotoxicity in the four cell types using an 80-compound library (76 unique) provided by the NTP (Table 1). The library contains 39 (37 unique) environmental compounds and drugs with reported developmental neurotoxicity (DNT) and/or neurotoxicity (NT) activity (Table 1, (Crofton et al., 2011)) as well as representatives of chemical classes (12 [11 unique] flame retardants [FRs], 17 PAHs) of interest to the NTP but with limited or unknown neurotoxicity information. In addition, the library contains six unclassified compounds, and six (five unique) negative control compounds for DNT/NT; included also are four compounds in duplicate to assess within assay reproducibility. Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., St. Louis, MO, USA) and stored at −20°C. Generally, stock concentrations were at ~20 mM except for six compounds prepared at lower concentrations (0.075 – 10 mM) due to limited solubility in DMSO. The compounds were provided coded and their identity was not known until the results were analyzed. Detailed information about the compound list, including the stock concentrations, is provided in Table 1.

iPSC culture and propagation

A subclone of the NCRM1 integration-free iPSC line (NIH Center for Regenerative Medicine), named XCL1 (XCell Science Inc.; Novato, CA, USA) was used for this study. The cell line was cultured as previously described (Lie et al., 2012; Zou et al., 2011) and

maintained in feeder-free conditions on Matrigel™ (BD Biosciences; San Jose, CA, USA) coated dishes using mTeSR™1 media (STEMCELL Technologies Inc.; Vancouver, Canada) following the manufacturer's protocols.

Generation of NSC, neurons and astrocytes

NSC, neurons, and astrocytes were developed using protocols that have been described previously (Shaltouki et al., 2013; Swistowski et al., 2009; Swistowski et al., 2010). In brief, NSC derived from XCL1 iPSC lines were cultured on Matrigel™ coated dishes in Neurobasal® medium supplemented with 1% nonessential amino acids, 1% GlutaMAX™, 1 × B-27®, and 10 ng/mL bovine fibroblast growth factor (bFGF), and passaged using Accutase® (all obtained from Life Technologies; Carlsbad, CA, USA).

Neuronal differentiation was achieved by culturing NSC in Neuronal Induction Medium (XCell Science, Inc) at surface coated with 2 μg/mL poly-L-ornithine (Sigma-Aldrich) and laminin (10 μg/mL, Life Technologies) at a density of 40–50 k/cm^2 for 5–6 days until the cells became confluent. Then, cells were split with Accutase® and were plated onto new polyornithine/laminin coated dishes at 40–50 k/cm^2 in Neuronal Maturation Medium (XCell Science, Inc.) to continue differentiation to day 14.

Astrocyte differentiation from NSCs was also carried out on culture dishes or glass cover slips coated with poly-L-ornithine/laminin in Astrocyte Induction medium (XCell Science, Inc.). Medium was changed every other day and cells were passaged at least 3 times before day 15. On day 18, the media was changed to Astrocyte Maturation medium (XCell Science, Inc.) and maintained on that media up to day 35.

Quantification of differentiation efficiency was performed as described previously (Shaltouki et al., 2013). In brief, ten randomly chosen fields from two independent experiments were counted and averaged. Total number of cells was counted as the number of dapi-labeled nuclei while the Tuj/GFAP positive cells were counted by analyzing fluorescent images using Photoshop.

Immunocytochemistry

Immunocytochemistry and staining procedures were performed as described previously (Zeng et al., 2003). Briefly, cells were fixed with 4% paraformaldehyde for 20 minutes, blocked in blocking buffer (10% goat serum, 1% bovine serum albumin [BSA], 0.1% Triton X-100) for one hour followed by incubation with the primary antibody at 4°C overnight in 8% goat serum, 1% BSA, 0.1% Triton X-100. Appropriately coupled secondary antibodies, Alexa350-, Alexa488-, Alexa546-, Alexa594-, or Alexa633 (Molecular Probes; Carlsbad, CA, USA, and Jackson ImmunoResearch Lab Inc.; West Grove, PA, USA) were used for single or double labeling. All secondary antibodies were tested for cross reactivity and non-specific immunoreactivity. The following primary antibodies were used: Oct4 (ab19857, Abcam, 1:1250), TRA 1-81 (12-8883-80, eBioscience, 1:60), SOX1 (AB15766, Millipore, 1:250), Nestin (611658, BD Transduction Laboratories, 1:500), β-III tubulin (clone SDL. 3D10, T8660, Sigma-Aldrich, 1:1000), Map2 (M9941, Sigma-Aldrich,1:500), glial fibrillary acidic protein (GFAP) (Z0334, DakoCytomation, 1:2000) and gamma-aminobutyric acid (GABA) (AB8891, Abcam, 1:2000)Quantification of immunoreactive cells in culture was

performed by analyzing fluorescent images using Adobe Photoshop (San Jose, CA, USA) on a minimum of 5000 cells of at least 10 randomly chosen fields derived from 3 or more independent experiments. The number of Hoechst labeled nuclei on each image was referred to as the total cell number (100%).

Gene expression by microarray

RNA isolated from dopaminergic populations were hybridized to Illumina Human HT-12 BeadChip (Illumina Inc.; San Diego, CA, USA, performed by the Microarray core facility at the Burnham Institute for Medical Research, Novato, CA, USA). Array data processing and analysis was performed using Illumina BeadStudio software. The Illumina array data were normalized by the background method. The maximum expression value for each probe set of one gene was chosen as the expression value of this gene. A differentially expressed gene was defined if the gene showed a 2-fold expression change between any two samples. All cell line correlations were a measure of Pearson's rho implemented in SAS (SAS Institute Inc.; Cary, NC, USA).

MTT assay

For each study, cells of the appropriate cell type (1×10^5 cells per well) were plated in 96-well plates containing 100 μL of each cell type appropriate medium. After 24 hours at 37°C (5% CO_2) to allow attachment, the cells were exposed to compounds for 24 hours, also at 37°C (5% CO_2). For screening the 80-compound library, cells were exposed to each compound at 10 and 100 μM, in duplicate. In a follow-up experiment, cells of each type were exposed to four compounds – valinomycin, 3,3′,5,5′-tetrabromobisphenol, deltamethrin, triphenyl phosphate – at 1, 10, and 100 μM in duplicate for 24 hours, with each test performed in triplicate. In a third study, the cytotoxicity of rotenone at 1 and 10 μM in duplicate was determined in NSC derived from seven additional iPSC lines established from both males and females, as well as in the XCL-1 line. For each study, at the end of the 24-hour exposure period, cell viability was evaluated using the MTT assay, as described previously (Peng et al., 2002). Briefly, MTT tetrazolium salt (5 mg/mL) was added to each well, and incubation was continued for two hours at 37°C. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with DMSO. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices; Sunnyvale, CA, USA). Cell survival was expressed as absorbance relative to that of untreated controls.

Luciferase-based assay in neural reporter line

GFAP-nanoluc astrocytes and MAP2-nanoluc neurons were generated as previous described (Pei et al., 2015). Determination of Nanoluc luciferase activity was measured using the Nano-Glo Assay System following manufacturer's protocol (Promega Corporation; Madison, WI, USA). In brief, after compound/DMSO treatment, culture media was completely removed and 100 μL of Nano-Glo Assay Reagent was added to each well in the 96-well culture plate for an incubation period of 5 min at 37°C to lyse the cells. Then, the reagent from each well was transferred to a new 96-well plate to measure the luciferase activity using a PerkinElmer Fusion-alpha-FP-HT universal microplate analyzer (PerkinElmer Inc.; Waltham, MA, USA).

Statistical Analysis

The 80 compound MTT data were analyzed using a standard ANOVA (Team, 2012). The data were subset by cell type (astrocyte, iPSC, neuron, NSC) and concentration (10 or 100 μM) and then tested for differences between control and dose (i.e., control vs 10 μM or control vs 100 μM) for each compound. Because we were interested in cytotoxic effects only, situations where there was an increase in MTT were excluded from consideration. Furthermore, compounds that exhibited a significant decrease in MTT at 10 but not 100 μM were classified as negative in this study. In a separate analysis to determine if there were significant differences across cell types, the data were first normalized by dividing each dose value by the mean value of the control replicates located within the same plate. Next, the data were subset by concentration (10 or 100 μM) and tested for differences between cell types. In both cases, significance was set at $p<0.01$. To produce the heat map, chemical-cell type per dose percent differences were clustered using the heatmap.2 function within the program R 3.1.2 (Team, 2012). Unsupervised hierarchical clustering was performed using Euclidean distance as the similarity metric and Ward's method for assembling clusters.

For the follow-up experiments, all data are expressed as mean ± standard error of the mean (S.E.M.) for the number (n) of independent experiments performed. Differences among the means for all experiments described were analyzed using one- or two-way analysis of variance. Newman-Keuls post-hoc analysis was employed when differences were observed by analysis of variance testing at $p<0.01$.

Results

Generation of purified populations of neural cells from iPSC

We have previously reported methods of generating a homogeneous population of NSC and subsequently differentiating them from multiple human ESC/iPSC into pure populations of neurons and astrocytes that are suitable for high throughput assays (Efthymiou et al., 2014; Han, 2009; Liu et al., 2013; Shaltouki et al., 2013). In this study, we generated NSC, neurons, and astrocytes from the well-characterized integration-free iPSC line XCL1 (Shaltouki et al., 2013; Swistowski et al., 2009; Swistowski et al., 2010) and used these isogenic cells for all assays unless otherwise described.

The quality of our iPSC and their neural derivative was routinely assessed by expression of cell-specific markers. Figure 1 shows the schema/timeline of our neural differentiation system and representative images of the expression of various markers by immunocytochemistry. For example, pluripotency markers Oct4 and Tra-1-81 were expressed in undifferentiated cells (Fig. 1A) while the NSC population uniformly expressed the NSC markers Sox1 and nestin (Fig. 1B). Using a 14-day neuronal differentiation protocol, we generated a pure population of neurons (Swistowski et al., 2009) as more than 95% of total cells expressed the neuron-specific markers β-III tubulin and Map2 by immunocytochemistry (Fig. 1C–D), while no or few astrocytes were identified in the neuronal culture (data not shown). The majority of the neurons expressed GABA receptor (Fig. 1E). For astrocyte differentiation, we used a 35-day differentiation protocol by which

>90% of the cells were expressing GFAP (Fig. 1F), with no or few neurons in the culture (Shaltouki et al., 2013).

Microarray analysis of individual cell types toward developing a cell type specific panel of markers

To further ensure the quality of our iPSC and their neural derivatives, we performed a whole genome expression profile of the four cell types. We have previously analyzed gene expression of many ESC/iPSC lines and their neural derivatives by microarray and have identified stage-specific markers for NSC, neurons, and astrocytes (Momcilovic et al., 2014). Based on our previous analysis and the literature, we have developed a panel of 10 to 20 cell-type specific genes for each NSC, neuron, and astrocyte population which was examined in the cells used in this study. As seen in Fig. 2, each cell population expressed the genes that were specific for that particular cell type. For example, the NSC population expressed all NSC genes at a high level, but not the genes representative of neurons or astrocytes. Likewise, the neuronal population had high levels of expression of many neuron-specific genes including DCX as well as Neurog1 and 2, whereas these markers were not expressed in NSC or astrocytes. Similarly, many astrocyte genes including GFAP and CD44 were expressed in the astrocyte population but not in the NSC or neuron populations.

Overall, our gene expression profiling of each cell type was similar to previously reported iPSC and their neural derivatives (Momcilovic et al., 2014), indicating that our differentiation protocol using off-the-shelf cell products provided suitable cells applicable for *in vitro* toxicity testing.

Response of iPSC and their neural derivative to the 80 compounds

One goal of this study was to demonstrate the potential utility of using human iPSC-derived neural cells for neurotoxicity assays. To accomplish this, we screened 80 drugs and environmental compounds, 39 (37 unique) with known neurotoxic potential, for cytotoxicity on iPSC and their isogenic neural derivatives. To reduce well-to-well variability, we grew NSC, neurons, and astrocytes in bulk culture before transferring to 96-well plates for assays. The primary screen was performed in duplicate at two concentrations (10 and 100 μM), and cell viability was measured using the MTT assay after 24 hours of compound exposure.

We defined a compound to be cytotoxic when it significantly reduced the conversion of MTT to formazan at $p<0.01$. In addition, we required that compounds inducing a significant reduction at 10 μM also induced a significant reduction at 100 μM. Based on this approach, of the 80 (76 unique) compounds tested, 50 compounds (48 unique) were active in at least one cell type; by cell type, 32, 38, 46, and 41 compounds induced significant cytotoxicity in iPSC, NSC, neurons, and astrocytes, respectively. Thus, the fewest number of compounds was significantly cytotoxic in iPSC while the greatest number was significantly cytotoxic in neurons.

Fig. 3 indicates the distribution of active compounds across the four cell types at 10 and 100 μM; six compounds (benzo[g,h,i]perylene; carbamic acid, butyl-,3-iodo-2-propynyl ester; deltamethrin; methyoxyethanol; fluorine; and rotenone) were cytotoxic in all four cell types at both 10 and 100 μM while 22 compounds were active in all four cell types at 100 μM

only; these included acetylsalicylic acid; acrylamide; L ascorbic acid; benzo[e]pyrene; captan, carbaryl; dichlorodiphenyltrichloroethane (DDT); deltamethrin; dieldrin; di(2-ethylhexyl) phthalate; 1-ethyl-3-methylimidazolium diethylphosphate; D-glucitol; 2,2′,4,4′,5,5′-hexabromodiphenyl ether; heptachlor; 1-methyl-4-phenylpyridinium iodide; naphthalene; phenobarbital sodium salt; phenol, isopropylated, phosphate (3.1); 6-propyl-2-thiouracil; 3,3′,5,5′-tetrabromobisphenol A; triphenyl phosphate; and valinomycin. A heat map organized by cell type and compound concentration, with compounds ranked by total cytotoxic activity (i.e., extent of cytotoxicity aggregated across cell types and concentrations) is provided in Figure 3. The compound with highest efficacy was benzo(g,h,i)perylene, followed by deltamethrin, fluorine, and rotenone.

In terms of the four sets of duplicate compounds, both copies of deltamethrin affected all four cell types at 100 μM, and either affected all four cell types or NSC and astrocytes only at 10 μM. Both copies of triphenyl phosphate had no effect at 10 μM, but one copy affected all cell types while the other copy affected iPSC and astrocytes only at 100 μM. One copy of methyl mercuric chloride did not affect any cell type whereas the other copy was cytotoxic at 10 and 100 μM in astrocytes. Similarly, one copy of saccharin sodium salt hydrate had no effect on any cell type, where the other copy was cytotoxic in neurons at 10 and 100 μM.

The analysis of the 80-compound data across cell types indicated that among the six compounds cytotoxic at 10 μM in all four cell types (deltamethrin; benzo[g,h,i]perylene; carbamic acid, butyl-,3-iodo-2-propynyl ester; fluorine; methyoxyethanol; and rotenone), only benzo[g,h,i]perylene exhibited a level of cytoxicity that depended on cell type ($p = 0.0097$), with astrocytes being less affected than the other three cell types. At 100 μM, of the 29 compounds active in all four cell types (Figure 3), three only (dieldrin, 2,2′,4,4′,5,5′-hexabromodiphenyl ether, and phenobarbital sodium salt) exhibited significant difference among cell types in the magnitude of cytotoxicity ($p<0.01$). In all cases, astrocytes exhibited significantly less cytotoxicity.

Dose response of a selected subset of toxic compounds

To extend the concentration response data and better evaluate assay reproducibility, we re-tested four compounds that were cytotoxic to all four cell types. A 1 μM concentration was tested in addition to the 10 and 100 μM concentrations and the assays were performed in triplicate. The compounds selected were: 1) valinomycin, a K+ Selective ionophoric cyclodepsipeptide; 2) 3,3′,5,5′-tetrabromobisphenol, a flame retardant for plastics, paper, and textiles; 3) deltamethrin, an insecticide that is a potent inhibitor of calcineurin; and 4) triphenyl phosphate, a relatively new flame retardant proposed as a replacement for brominated flame retardant, which were phased out due to concerns about developmental neurotoxicity. The resulting data for each cell type are shown in Figure 4. None of the compounds were significantly cytotoxic in any cell type at 1 μM while all four compounds were significantly cytotoxic at 100 μM. Depending on the compound and the cell type, significant cytotoxicity occurred at 10 μM. These results are consistent with those obtained in the first study and demonstrate the reproducibility of cytotoxicity data for these compounds using iPSC-derived neural cells.

The assay works in multiple iPSC lines with a different allelic background

We next examined how multiple iPSC lines, including lines derived from males and females, would respond to the same cytotoxicant. To do so, we tested seven additional iPSC lines that our laboratory has generated and characterized. These include four lines generated from females and three lines generated from males (XCL1 is a male line). To simplify the experiment, we ran the assay with only one widely used compound (rotenone, a mitochondrial toxicant) in one cell type (NSC) for all lines. Since we observed significant cytotoxicity for rotenone in NSC at 10 μM in the primary screen, we screened this compound at 1 and 10 μM in these cell lines as well as with the control line XCL1. No apparent cytotoxicity as measured by the MTT assay was observed in any of the NSC lines (including the control line XCL1) exposed to rotenone at 1 μM, whereas significant cytotoxicity (~60%) was induced ($p<0.01$) in all lines at 10 μM (Figure 5). Differential cytotoxicity was not observed between the NSC lines, indicating the lack of a genetic or gender difference in the sensitivity of these eight lines to rotenone.

Luciferase-based cytotoxicity assessment in neural reporter lines

To extend the utility of our screening system, we developed a panel of luciferase-based neural reporter iPSC lines. Specifically, we generated a neuron reporter by tagging the luciferase gene into the endogenous Map2 loci (Map2-nanoluc), and an astrocyte reporter by tagging the luciferase gene into the endogenous GFAP loci (GFAP-nanoluc). We have previously validated lineage-specific expression of these two reporter lines during lineage-specific differentiation (i.e., the luciferase activity in the Map2-nanoluc line reflects the percentage of neurons in the neuronal differentiation culture while the luciferase activity in the GFAP-nanoluc line reflects the percentage of astrocytes in the astrocyte differentiation culture)(Pei et al., 2015).

To determine if luciferase activity can serve as cytotoxicity readout upon toxin challenge, we tested two compounds, 3,3′,5,5′-tetrabromobisphenol and triphenyl phosphate, which were significantly cytotoxic to neurons and astrocytes as measured by the MTT assay in our primary and secondary screens (Figure 4), in both GFAP-nanoluc and Map2-nanoluc reporter lines. To do this, we plated either mature GFAP-nanoluc astrocytes or Map2-nanoluc neurons differentiated from our reporter iPSC lines into 96-well plate and treated them with the compounds at 100 μM or DMSO only as the negative control ($n>4$). As seen in Figure 6, 3,3′,5,5′-tetrabromobisphenol at 100 μM reduced the luciferase signal in astrocytes and neurons by ~80% and ~40%, respectively, while triphenyl phosphate at 100 μM reduced the luciferase signal in astrocytes and neurons by ~90% and ~70%, respectively. These results are similar to the toxicity measured by the MTT assay (see Figure 4).

Discussion

An emerging field of investigation in stem cell research is the use of iPSC-derived cell populations for toxicological testing. Although proof-of-concept studies have been reported that evaluated the toxic effects of compounds to stem cells and their differentiated derivatives (Ebert and Svendsen, 2010; Gupta et al., 2009; van Dartel and Piersma, 2011), several issues need to be addressed before such assays can be used routinely to reduce or

replace the expensive and time-consuming *in vivo* laboratory animal tests. First, we need to establish reproducible and reliable differentiation procedures for the generation of differentiated cells in required purity and in sufficient numbers, as well as in an assay ready format. Second, we need to develop assays that are reliable and relevant for a wide range of chemicals. Third, we need to increase assay sensitivity and throughput; one possible approach is through the use lineage-specific reporters. Here, we characterized the feasibility of using iPSC-derived neural cells for neurotoxicity assays. Using an iPSC-based step-wise neural differentiation system (iPSC→NSC→neurons or astrocytes), we tested the responses of iPSC-derived neural populations (NSC, neurons, astrocytes) to 80 compounds including known NTs, known DNTs, and environmental compounds of potential concern (e.g., flame retardants, PAHs). Based on these initial data, 23 of the 37 unique compounds classified as "known" DNT/NT compounds (Table 1) were cytotoxic in at least one of the cell types tested (Figure 3). The lack of activity by the other 14 DNT/NT compounds is not surprising since the selected compounds cover multiple aspects of DNT and NT. For example, some of the compounds in this category were chosen specifically due to their effects on neuronal firing (e.g., permethrin), effects on neurobehavior in zebrafish (e.g., chlorpyrifos), or based on other *in vivo* findings (e.g., toluene, hexachlorphene). Among the 36 (35 unique) compounds not classified as DNT/NT positive or negative compounds (i.e., those classified as flame retardants, PAHs, or other), 22 were cytotoxic in at least one cell type.

Some variability in activity was noted among the four compounds tested in duplicate (deltamethrin, triphenyl phosphate, saccharin sodium, methyl mercuric chloride). We also noted cytotoxic activity induced by the presumed DNT/NT negative control compounds acetaminophen, acetylsalicylic acid, D-glucitol, L-ascorbic acid, and saccharin sodium salt hydrate at one or more of the concentrations tested in one or more cell types, which might reflect their intrinsic biological activity or false positive responses in this *in vitro* model system. The lack of consistency for some compounds suggests the need for more in-depth characterization of the assay in terms of exposure duration, numbers of replicate samples per concentrations, numbers of concentrations tested, and numbers of independent cell lines for each cell type.

Based on the initial screen, we selected four cytotoxic compounds (valinomycin, 3,3′,5,5′-tetrabromobisphenol, deltamethrin, triphenyl phosphate) and retested them for further evaluation. This subset demonstrated good reproducibility in all four cell types and obtained similar results to those seen in the initial screen. We showed also that our assay system worked well in multiple iPSC lines including lines developed from humans of both genders. In addition, we demonstrated that we could simplify and make more efficient the assay by using luciferase lineage-specific reporters rather than using the MTT assay.

We have previously published methods of generating central nervous system (CNS) cells including NSC, neurons, and astrocytes from iPSC (Swistowska et al., 2010; Swistowski et al., 2009). To adapt our culture system for screening, we optimized the differentiation processes of generating pure populations of differentiated cells (e.g., neurons and astrocytes) for 96-well plates, a format which is suitable for low to medium throughput screening. To provide consistency and reduce well-to-well variability of each batch of cells, we used NSC as a stable intermediate to produce neurons and astrocytes, as NSC allow for storage and

good viability after cryopreservation. The cells can be readily plated into 96- or higher well formats for assays, and the same NSC can be used to make multiple subtypes of neurons or astrocytes or other CNS cells including oligodendrocyte precursors. An additional advantage of using NSC as gateway cells is the significant reduction of differentiation time period, which is critical for developing and validating assays for screening. Although it is out of the scope of this study, our stage specific differentiation process allows for automation and scale-up of cell numbers for high throughput screening.

One important finding of this study is that our iPSC-based screen can identify cell-type specific toxicity. Unlike experimental rodents, humans are outbred and have diverse genetic backgrounds. To minimize the effect of allelic variability, which might make it difficult to interpret different phenotypic effects seen using different cell types, we elected to screen compounds using isogenic cells (i.e., the various cell types (NSC, neurons and astrocytes) that were derived from the same iPSC line. Of the 80 compounds screened, 50 induced significant cytotoxicity in at least one cell type at 100 μM or at 10 and 100 μM. More chemicals were significantly cytotoxic in neurons, followed by astrocytes, NSC, and iPSC. However, for chemicals that were cytotoxic in all four cell types but exhibited cell-type significant differences, astrocytes exhibited the least induced cytotoxicity. These findings show that different groups of compounds have different activity profiles with some being active at both low and high concentration in all cell types (e.g., deltamethrin, fluorine), while others are selectively active in only certain cell types (e.g., diazepam, dibenz[a,h]anthracene, and tebuconazole appear to affect only neurons). This has important implications in identifying potential mechanisms of neurotoxicity associated with different compounds and/or classes of compounds. Furthermore, the clustering method provides clues about how some of these compounds with unknown neurotoxic potential may behave similarly to other drugs or known neurotoxicants. For example, might the PAH benzo[g,h,i]perylene have an underlying mechanism similar to diazepam?

Equally important is that our assay systems (differentiation procedures and toxicity assay) work well in multiple iPSC lines of both male and female lines. Although we did not test all 80 compounds in all cell lines at all stages of differentiation, of the eight iPSC lines (4 male and 4 female lines) we tested, a representative compound (rotenone) had a similar toxic effect in all eight lines at the NSC stage. This result, based on this limited number of NSC lines, did not detect rotenone-induced difference in sensitivity and suggests that our assay is robust and reliable, and suitable for further development using panels of cell lines.

Another unique advantage of an iPSC-based screen is the ability to engineer iPSC lines. For example, iPSC can be engineered in multiple ways to investigate how genetic alterations modulate physiological and disease processes. These engineered tools can be further applied in disease pertinent cellular lineages as well as developing isogenic and reporter lines (Zeng et al., 2014; Zhu et al., 2011). Our neural lineage-specific luciferase reporters will simplify the cytotoxicity assay using neurons or astrocytes and will also allow for an evaluation of the ability of chemicals to adversely affect the differentiation process.

In summary, although laboratory animal models have been and will continue to be important for neurotoxicity assays, there are critical differences in nervous system development

between humans and rodents (Rice and Barone Jr, 2000). Because of this, the availability of human iPSC and their differentiated derivatives is critical for properly understanding the human nervous system biology including neurotoxicity and development neurotoxicology (Hoelting et al., 2014; Hou et al., 2013; Maldonado-Soto et al., 2014). Our results demonstrate that it is important to test toxicity on specific cells types as significant differences in responses were seen between different neural cells (e.g., neurons versus astrocytes). We chose a widely available iPSC line for our assays and provide whole genome expression data so others can repeat and compare with our results. Finally by using an isogenic line we can easily add other neural lineages including oligodendrocytes and the peripheral nervous system (PNS) cells to future studies that will focus on assessing a more comprehensive concentration-response and time-course for these compounds in order to better determine relative potency and kinetics of effect.

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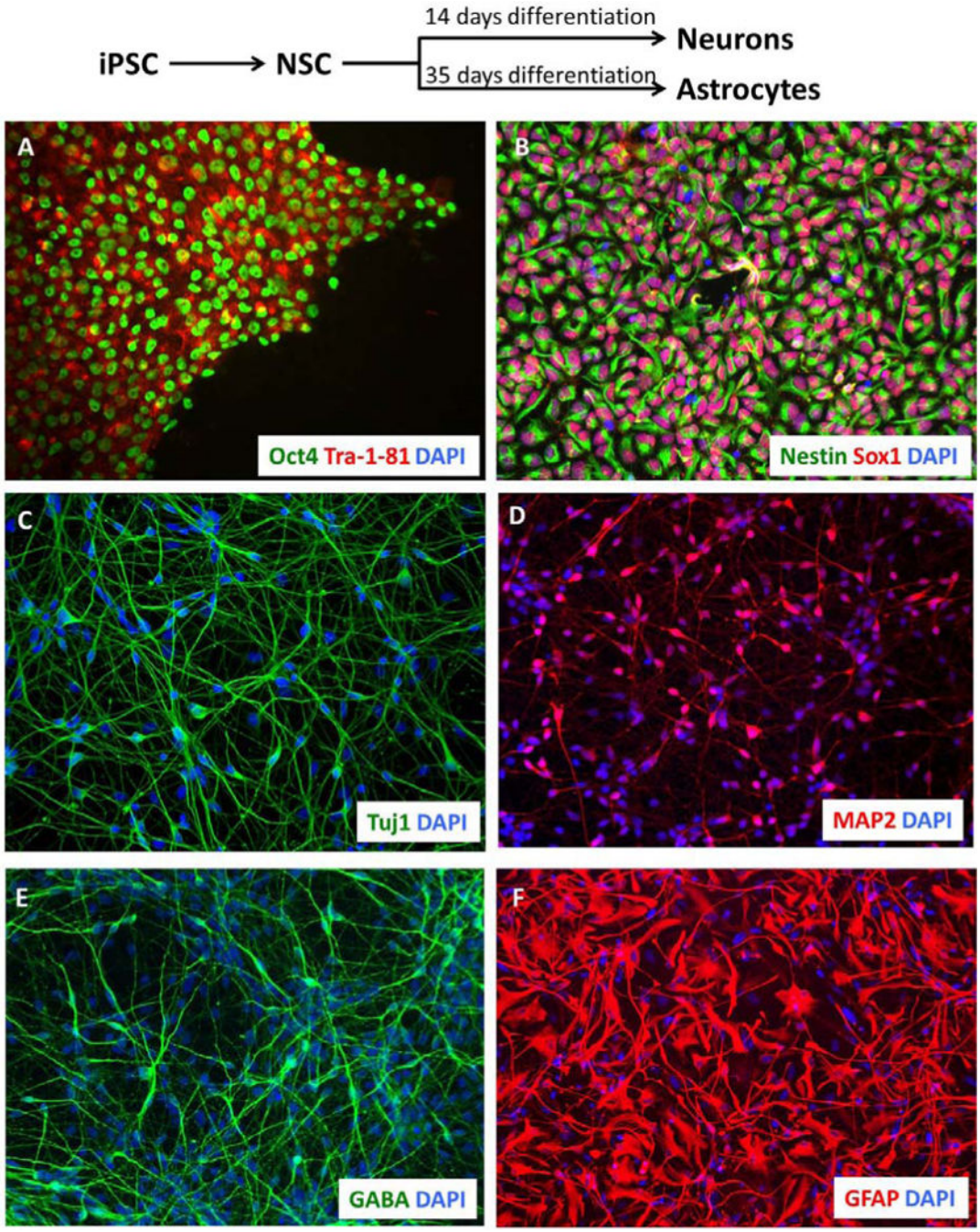

Fig. 1. Generation of pure populations of NSC, neurons, and astrocytes from the human iPSC line XCL1

The human iPSC line XCL1 was analyzed by immunofluorescence before and during neural differentiation. At the iPSC stage, XCL1 showed normal expression of pluripotency markers Oct4 and Tra 1–80 before differentiation (A). Then, XCL1 iPSC was directed to NSC cells where the NSC markers Sox1 and Nestin were detected in more than 95% of the cells (B). Next, these progenitors were further differentiated into either a pure population of forebrain type neurons (C–E) or astrocytes (F). After neuronal differentiation, more than 95% of the population expressed the neuronal specific markers β-III tubulin (Tuj1) (C), Map2 (D) and

GABA receptor (D & E). Similar purity was detected after astrocyte differentiation using the marker GFAP (F). DAPI was used to label the nuclei and scale bar is 100 μm.

SYMBOL	NSC	NEURONS	ASTROCYTES	Description
ADM	13434	57	1285	
ALPL	2357	240	791	
C1orf135	720	74	258	
CMTM8	1384	33	529	
FAM46B	564	-9	214	
FHOD1	484	81	206	
HIST1H2BH	1362	98	140	
ITGB3BP	195	10	48	
LIN28B	5177	358	137	
NEFH	513	73	174	
NMU	8367	169	434	
PHLDB2	784	2	284	
PLAGL1	468	90	55	NSC
ROR2	998	131	259	
SDC2	4054	315	1418	
SMO	1119	471	750	
SNORA67	642	468	445	
SOX2	14404	11484	11107	
SPRYD5	1217	292	48	
TFPI2	738	22	104	
TMEM88	937	313	49	
TRIM48	811	140	29	
UCK2	1005	258	493	
USP44	299	34	4	
ZIC2	13599	622	618	
ATP10B	2	489	165	
DCX	467	7918	1026	
GREM2	11	685	182	
LHX1	15	1878	36	
LOC150568	284	640	317	
MAB21L2	2	2660	7	
MAP6	1266	4210	1004	
MYT1	70	8591	578	
NEUROD1	53	328	190	
NEUROG1	34	570	137	NEURON
NEUROG2	207	8367	131	
OPRK1	245	450	96	
PLXNA2	23	780	328	
POU4F2	-5	1386	69	
RGS1	8	13	9	
SEMA5A	212	482	282	
SLC17A6	12	2357	161	
SLC17A8	-15	316	76	
TUBB3	20296	54556	35715	
AQP4	-23	-5	1082	
CCL2	396	63	2991	
CD44	29	43	8716	
CRYAB	-21	5	9661	
GFAP	-2	1	30956	
HOPX	-14	96	777	
LGALS3	33	8	1203	ASTRO
NFIA	-43	981	2110	
NFIX	-6	720	7849	
PMP2	12	606	3186	
PRRX1	23	19	3973	
S100A6	137	142	1952	
SPARCL1	1092	40	13584	
TNC	164	589	4838	

Fig. 2. Gene expression analysis of individual cell types and developing a cell type specific panel of markers

The quality of our iPSC and their neural derivatives were analyzed by whole genome expression profile. A panel of genes was selected from the microarray as stage-specific markers for iPSC, NSC, neurons, and astrocytes. In comparison of each gene in all 4 stages, expression increased (red) and decreased (green) is indicated in the heat map.

Fig. 3. Heat map - Response of iPSC and their neural derivatives to 80 compounds

80 compounds, some with known neuotoxicity or developmental neurotoxicity potential, were tested for cytotoxicity in XCL1 iPSC, NSC, neurons, and astrocytes. The assay was performed in a 96-well plate format and cells were treated with each compound at 10 and 100 μM, in duplicate, for 24 hours. At the end of the exposure period, the MTT assay was used to measure cell viability. The compounds are organized by most active across the concentrations tested and the cell types based on summing the extent of cytotoxicity. Blue represents cytotoxicity in 25% increments relative to control.

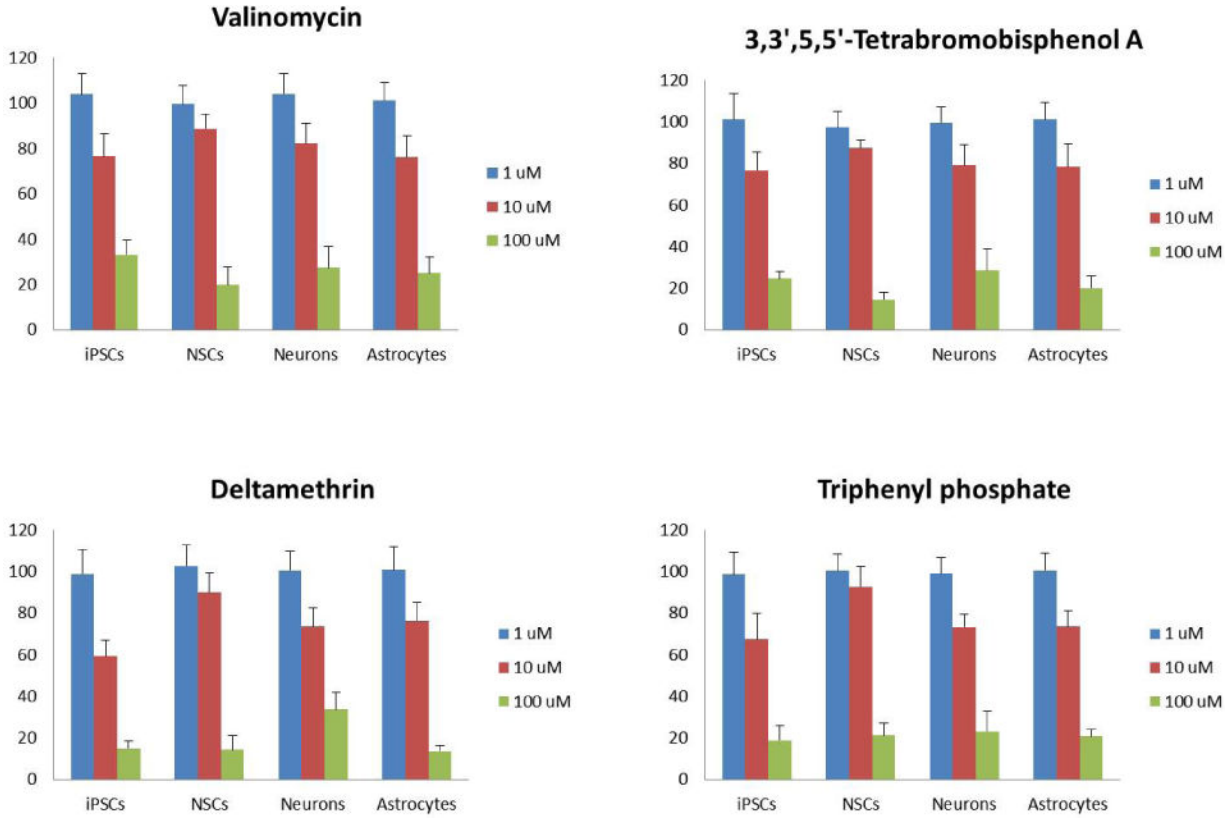

Fig. 4. Concentration response of four selected compounds on iPSC-derived neural cells

Four compounds – valinomycin, 3,3′,5,5′-tetrabromobisphenol, deltamethrin, triphenyl phosphate – were tested at 1, 10, and 100 μM for cytotoxicity in XCL-1 iPSC, NSC, neurons, and astrocytes. Each test was performed in triplicate and the MTT assay was used to determine viability after 24 hours of treatment. Bars represented the percentage of viable cells normalized by the control wells (DMSO only). Error bars represent standard deviation.

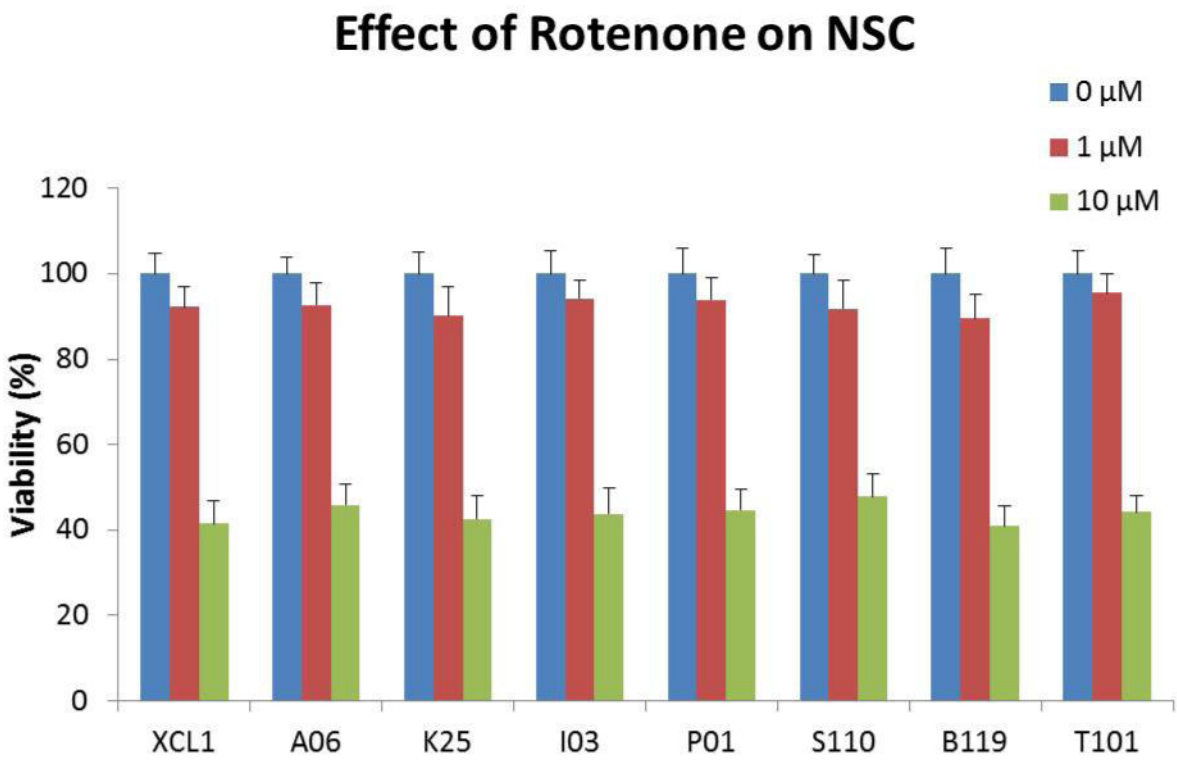

Fig. 5. Effect of rotenone on multiple NSC lines

The cytotoxicity of rotenone was tested in NSC derived from seven additional iPSC lines of lines isolated from both males and females and the control XCL-1 line. Cells were exposed to the compounds at 1 and 10 µM. Cell viability was calculated via MTT assay after 24 hour of treatment. Data are expressed as relative percentage of cell viability using the DMSO solvent control wells to normalize the rotenone-treated samples. Error bars represent standard deviation.

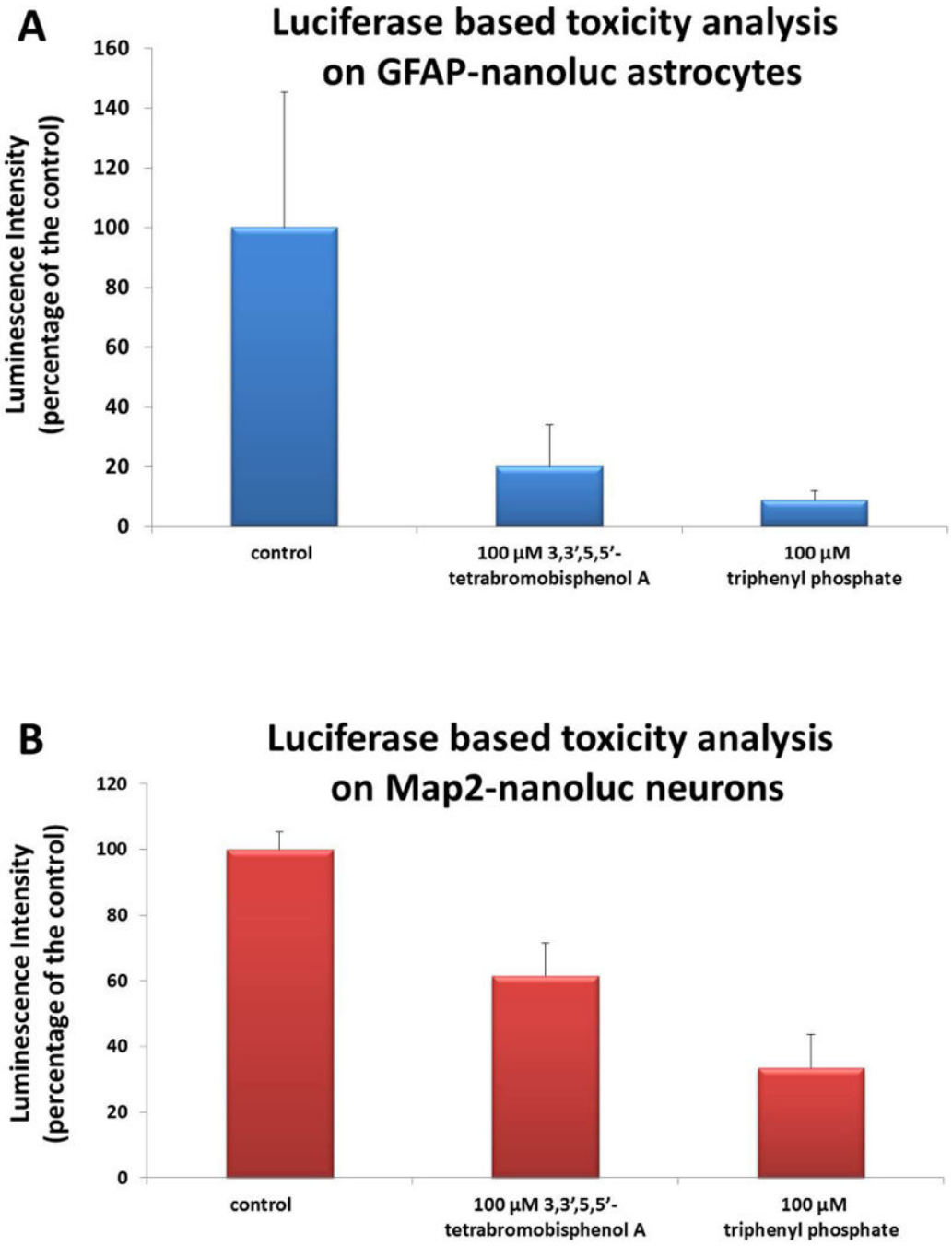

Fig. 6. Toxicity tests on selected compounds using a luciferase assay in neurons or astrocytes derived from the reporter iPSC lines

3,3′,5,5′-tetrabromobisphenol A and triphenyl phosphate, both at 100 µM were tested for cytotoxicity using the GFAP-nanoluc astrocytes (A) and Map2-nanoluc neurons (B) luciferase assays. The luciferase levels per well were measured from the nanoluc reporter signal from each reporter lines and were used to calculate the cytotoxicity of each compound on astrocytes or neurons (n>4).

Table 1

Compound Library

Compound	CASRN	Molecular Weight	Aliquot Stock Conc. (mM)	Supplier	Category	References
1-methyl-4-phenylpyridinium iodide	36913-39-0	297.13	20.33	Sigma-Aldrich	DNT/NT	(Desai et al., 1996; Johannessen, 1991; Notter et al., 1988)
2-Methyoxyethanol	109-86-4	76.09	20.92	Sigma-Aldrich	DNT/NT	(Nelson et al., 1984; Regulska et al., 2010)
3,3′-Iminodipropionitrile	111-94-4	123.16	20.41	TCI America	DNT/NT	(Crofton et al., 1993; Moser and Boyes, 1993; Peele et al., 1990)
5-Fluorouracil	51-21-8	130.08	20.63	Sigma-Aldrich	DNT/NT	(Mustafa et al., 2008; Sucker et al., 2002; Yamaguchi et al., 2009)
6-Hydroxydopamine hydrochloride	28094-15-7	205.64	20.08	Sigma-Aldrich	DNT/NT	(Bradbury et al., 1986; Glinka et al., 1997)
6-Propyl-2-thiouracil	51-52-5	170.23	20.53	Sigma-Aldrich	DNT/NT	(Axelstad et al., 2008; Gilbert and Paczkowski, 2003; Shiraki et al., 2014)
Acetic acid, manganese(2+) salt	638-38-0	173.03	20.29	Sigma-Aldrich	DNT/NT	(Normandin et al., 2002; Ordonez-Librado et al., 2010; Sistrunk et al., 2007)
Acrylamide	79-06-1	71.08	20.06	Sigma-Aldrich	DNT/NT	(Erkekoglu and Baydar, 2014; Exon, 2006; Pennisi et al., 2013)
Aldicarb	116-06-3	190.26	20.16	Sigma-Aldrich	DNT/NT	(Moser, 1999; Palumbo et al., 2001; Risher et al., 1987)
Bisphenol A	80-05-7	228.29	20.38	Sigma-Aldrich	DNT/NT	(Miyagawa et al., 2007; Palanza et al., 2008; Patisaul et al., 2007)
Bis(tributyltin)oxide	56-35-9	596.11	1.00	Sigma-Aldrich.	DNT/NT	(Elsabbagh et al., 2002; Gerasimiak et al., 1994)

Compound	CASRN	Molecular Weight	Aliquot Stock Conc. (mM)	Supplier	Category	References
Carbaryl	63-25-2	201.22	19.85	Sigma-Aldrich	DNT/NT	(Moser, 1995; Sachana et al., 2003; Wang et al., 2014)
Chlorpyrifos (Dursban)	2921-88-2	350.59	20.04	Sigma-Aldrich	DNT/NT	(Hernandez et al., 2015; Howard et al., 2005; Slotkin et al., 2012; Yen et al., 2011)
Colchicine	64-86-8	399.44	19.81	Sigma-Aldrich	DNT/NT	(Mundy and Tilson, 1990; Pitts et al., 1991; Zaniani et al., 2013)
Deltamethrin *	52918-63-5	505.20	20.68	Chem Service, Inc.	DNT/NT	(Eriksson and Fredriksson, 1991; Harrill et al., 2008; Shafer et al., 2008)
Di(2-ethylhexyl) phthalate	117-81-7	390.56	19.79	Unknown	DNT/NT	(Sui et al., 2014; Wu et al., 2014)
Diazepam	439-14-5	284.74	20.25	TCI America	DNT/NT	(Batool and Haleem, 1997; Hu et al., 2006; Ouardouz and Sastry, 2006)
Dichlorodiphenyltrichloroethane (DDT)	50-29-3	354.49	20.12	Sigma-Aldrich	DNT/NT	(Ferguson and Audesirk, 1990; Shinomiya and Shinomiya, 2003; Zhao et al., 2012)
Dieldrin	60-57-1	380.91	19.71	Sigma-Aldrich	DNT/NT	(Kanthasamy et al., 2005; Richardson et al., 2006)
Diethylstilbestrol	56-53-1	268.35	19.87	Sigma-Aldrich	DNT/NT	(Kaitsuka et al., 2007; SanMartin et al., 1999; Wilkinson and Herdon, 1982)
Heptachlor	76-44-8	373.32	20.07	Pfaltz & Bauer, Inc.	DNT/NT	(Kirby et al., 2001; Miller et al., 1999; Moser et al., 2001)
Hexachlorophene	70-30-4	406.90	19.96	Sigma-Aldrich	DNT/NT	(Itahashi et al., 2015; Prasad et al., 1987; Shuman et al., 1974, 1975)

Compound	CASRN	Molecular Weight	Aliquot Stock Conc. (mM)	Supplier	Category	References
Hydroxyurea	127-07-1	76.05	20.22	Sigma-Aldrich	DNT/NT	(Guan et al., 2015; Vorhees et al., 1979)
Lindane	58-89-9	290.83	19.88	Sigma-Aldrich	DNT/NT	(Boffa et al., 1995; Joy and Albertson, 1987; Nolan et al., 2012)
Manganese, tricarbonyl[(1,2,3,4,5-.eta.)-1-methyl-2,4-cyclopentadien-1-yl]-	12108-13-3	218.09	20.01	Sigma-Aldrich	DNT/NT	(Normandin et al., 2002; Sistrunk et al., 2007)
Methyl mercuric (II) chloride *	115-09-3	251.08	19.96	Sigma-Aldrich	DNT/NT	(Cooper and Kusnecov, 2007; Juang and Yonemura, 1975; Parran et al., 2001)
n-Hexane	110-54-3	86.18	19.95	Sigma-Aldrich	DNT/NT	(Bachmann et al., 1993; Tahti et al., 1997; Woehrling et al., 2006)
Parathion	56-38-2	291.26	20.22	Sigma-Aldrich	DNT/NT	(Adigun et al., 2010; Garcia et al., 2003; Levin et al., 2010)
permethrin	52645-53-1	391.29	19.89	Chem Service, Inc.	DNT/NT	(Carloni et al., 2013; Harrill et al., 2008; Shafer et al., 2008)
Phenobarbital	50-06-6	232.24	20.36	TCI America	DNT/NT	(Gutherz et al., 2014; Yanai et al., 1989)
Phenobarbital sodium salt	57-30-7	254.22	20.05	Ganes Chemicals Inc.	DNT/NT	(Rogel-Fuchs et al., 1992; Schnare and Lenzer, 1978)
Rotenone	83-79-4	394.42	20.22	Sigma-Aldrich	DNT/NT	(Cannon et al., 2009; Krug et al., 2013; Wu and Johnson, 2007)
Tebuconazole	107534-96-3	307.82	20.20	Bayer Agriculture Division	DNT/NT	(Heusinkveld et al., 2013; Moser et al., 2001)
Tetraethylthiuram disulfide (disulfiram)	97-77-8	296.54	19.99	Sigma-Aldrich	DNT/NT	(Lee and Peters, 1976; Wood et al., 2014)
Thalidomide	50-35-1	258.23	19.96	Sigma-Aldrich	DNT/NT	(Cavaletti et al., 2004; Qin et al., 2012; Vorhees et al., 2001)

Compound	CASRN	Molecular Weight	Aliquot Stock Conc. (mM)	Supplier	Category	References
Toluene	108-88-3	92.14	19.67	Sigma-Aldrich	DNT/NT	(MacPhail et al., 2012; Win-Shwe and Fujimaki, 2010; Yucel et al., 2008)
Valproic acid sodium salt	1069-66-5	166.19	20.08	Sigma-Aldrich	DNT/NT	(Chaudhary and Parvez, 2012; Martin and Manzoni, 2014; Rinaldi et al., 2008)
2- ethylhexyl diphenyl phosphate (EHDP)	1241-94-7	362.40	20.24	TCI America	FR	
2,2′,4,4′,5-Pentabromodiphenyl ether	60348-60-9	564.69	20.00	LKT Laboratories, Inc.	FR	
2,2′,4,4′,5,5′-Hexabromodiphenyl ether	68631-49-2	643.58	10.01	LKT Laboratories, Inc.	FR	
2,2′4,4′-Tetrabromodiphenyl ether	5436-43-1	485.79	20.03	LKT Laboratories, Inc.	FR	
3,3′,5,5′-Tetrabromobisphenol A	79-94-7	543.87	19.94	Albemarle	FR	
Isodecyl diphenyl phosphate	29761-21-5	390.45	20.08	Bayville Chemical Supply Co.	FR	
Phenol, isopropylated, phosphate (3:1)	68937-41-7	452.52	19.99	Amfinecom Inc.	FR	
tert-Butylphenyl diphenyl phosphate	56803-37-3	382.39	20.11	Ubichem	FR	
Tricresyl phosphate	1330-78-5	368.36	19.98	Sigma-Aldrich	FR	
Triphenyl phosphate *	115-86-6	326.28	19.91	Sigma-Aldrich	FR	
Tris(2-chloroethyl) phosphate	115-96-8	285.49	19.86	Sigma-Aldrich	FR	
Acetaminophen (4-hydroxyacetanilide)	103-90-2	151.16	20.60	Sigma-Aldrich	NC	
Acetylsalicylic acid	50-78-2	180.16	19.90	Sigma-Aldrich	NC	
D-Glucitol	50-70-4	182.17	20.22	Sigma-Aldrich	NC	
L-Ascorbic acid	50-81-7	176.12	19.97	Sigma-Aldrich	NC	
Saccharin Sodium Salt hydrate *	82385-42-0	205.17	20.19	Sigma-Aldrich	NC	
1-ethyl-3-methylimidazolium diethylphosphate	848641-69-0	264.26	20.15	Sigma-Aldrich	Other	
2,3,7,8-Tetrachlorodibenzo-p-dioxin	1746-01-6	321.97	0.08	NCI Carcinogen Repository	Other	
Berberine chloride	633-65-8	371.81	20.13	Sigma-Aldrich	Other	
Captan	133-06-2	300.59	19.93	Sigma-Aldrich	Other	
Carbamic acid, butyl-, 3-iodo-2-propynyl ester	55406-53-6	281.09	19.92	Sigma-Aldrich	Other	
Valinomycin	2001-95-8	1111.32	20.02	Sigma-Aldrich	Other	

Compound	CASRN	Molecular Weight	Aliquot Stock Conc. (mM)	Supplier	Category	References
4-H-Cyclopenta(d,e,f)phenanthrene	203-64-5	190.24	20.36	Sigma-Aldrich	PAH	
Acenaphthene	83-32-9	154.21	20.42	Sigma-Aldrich	PAH	
Acenaphthylene	208-96-8	152.19	19.71	Sigma-Aldrich	PAH	
Anthracene	120-12-7	178.23	20.53	Sigma-Aldrich	PAH	
Benz(a)anthracene	56-55-3	228.29	20.06	Sigma-Aldrich	PAH	
Benzo(a)pyrene	50-32-8	252.31	20.32	Sigma-Aldrich	PAH	
Benzo(b)fluoranthene	205-99-2	252.31	19.62	Sigma-Aldrich	PAH	
Benzo(e)pyrene	192-97-2	252.31	19.89	Sigma-Aldrich	PAH	
Benzo(k)fluoranthene	207-08-9	252.31	19.77	Sigma-Aldrich	PAH	
Benzo[g,h,i]perylene	191-24-2	276.33	0.84	Sigma-Aldrich	PAH	
chrysene	218-01-9	228.29	9.44	Sigma-Aldrich	PAH	
Dibenz(a,h)anthracene	53-70-3	278.35	10.12	Sigma-Aldrich	PAH	
Dibenz[a,c]anthracene	215-58-7	278.35	20.07	Sigma-Aldrich	PAH	
Fluorene	86-73-7	166.22	20.25	Sigma-Aldrich	PAH	
Naphthalene	91-20-3	128.17	19.93	Sigma-Aldrich	PAH	
Phenanthrene	85-01-8	178.23	19.55	Sigma-Aldrich	PAH	
Pyrene	129-00-0	202.25	20.14	Sigma-Aldrich	PAH	

* compounds in duplicate.

Abreviations: CASRN = Chemical Abstract Services Registry Number, DNT/NT = developmental neurotoxicant/neurotoxicant; FR = flame retardant, Inc. = incorporated, NC = negative control for DNT/NT, PAH = polycyclic aromatic hydrocarbon.