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Anchoring a dynamic *in vitro* model of human neuronal differentiation to key processes of early brain development *in vivo*

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Abstract

We characterize temporal pathway dynamics of differentiation in an *in vitro* neurotoxicity model with the aim of informing design and interpretation of toxicological assays. Human neural progenitor cells (hNPCs) were cultured in differentiation conditions up to 21 days. Genes significantly changed through time were identified and grouped according to temporal dynamics. Quantitative pathway analysis identified gene ontology (GO) terms enriched among significantly changed genes and provided a temporal roadmap of pathway trends *in vitro*. Gene expression in hNPCs was compared with publicly available gene expression data from developing human brain tissue *in vivo*. Quantitative pathway analysis of significantly changed genes and targeted analysis of specific pathways of interest identified concordance between *in vivo* and *in vitro* expression associated with proliferation, migration, differentiation, synapse formation, and neurotransmission. Our analysis anchors gene expression patterns *in vitro* to sensitive windows of *in vivo* development, helping to define appropriate applications of the model.

Keywords

neuronal differentiation; brain development; developmental pathway dynamics

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Competing Interests

The authors have declared that no competing interests exist.

Supporting Information

Table S1. Lists of enriched pathways and associated genes among genes in cluster 1, 2, and 3 *in vitro*.

Table S2. List of enriched pathways among genes significantly changed through time *in vivo* and *in vitro*, only *in vivo*, or only *in vitro*.

Table S3. Lists of genes expressed (above the 50th percentile of gene expression) on D0 and D21 *in vitro*.

Figures S1–S9. Heatmaps of *in vivo* and *in vitro* gene expression in targeted pathways of interest

1. Introduction

1.1 Developmental neurotoxicity and public health trends

Disruption of the precisely timed regulation of critical processes in brain development can result in lifelong neuropathologies [1–3]. There is increasing evidence that environmental exposures to a diverse set of chemicals can result in cognitive deficits and contribute to neurodevelopmental disorders such as autism, attention deficit disorder, cerebral palsy and mental retardation [4–6]. The severe, well-characterized neurodevelopmental effects of gestational exposures to methylmercury [7] and alcohol [8] demonstrate the sensitivity of the developing brain.

Effects of toxicant exposures on brain development and function are dependent on developmental context. Brain regions that are actively developing at the time of an environmental exposure tend to be most susceptible to perturbation [1]. At the cellular level, the proliferation and differentiation status of cells can influence their susceptibility [9–13]. Furthermore, toxicant exposure can alter the process of differentiation itself, redirecting differentiating cells towards inappropriate neuronal subtypes. For example, methylmercury targets genes involved in promotion and regulation of differentiation [14], the potency of rotenone neurotoxicity is dependent on neuronal differentiation status [15], and several other toxicants have been shown to influence the regional identity adopted by differentiating neurons [16]. This evidence, along with the critical windows of susceptibility observed in human populations, demonstrates that the developmental processes occurring at the time of exposure influence the type and severity of the effect.

Given the context-specific nature of neurotoxic effects, it is essential to understand complex temporal dynamics of biological processes occurring in *in vitro* models used for neurotoxicity screening and to design toxicity tests with the specific biological context of the model in mind. In order to evaluate the neurotoxic effects of chemicals on specific processes of brain development, we must first determine the extent to which pathways of interest for neurodevelopment are active in the test models used and optimize exposure timing to target those pathways of interest. Toxicity testing paradigms will need to include multiple models and exposure at multiple timepoints that capture a range of processes important to brain development [17, 18].

Despite the clear public health need to prevent hazardous exposures during sensitive stages of development, the vast majority of chemicals in commerce remain untested for developmental neurotoxicity [19, 20]. Given the large number of chemicals that still need to be evaluated for potential hazard, the expensive and time consuming animal models that have long been the gold standard of developmental toxicology are insufficient for addressing this data gap [21]. Emerging high throughput and high content *in vitro* models offer promising alternatives. These *in vitro* models can be used to quantify toxicant-induced pathway perturbation to predict adverse developmental outcomes [22–24].

1.2 *In vitro* models for brain development

There has already been great progress in the development and optimization of *in vitro* models of neuronal differentiation and brain development. A diverse set of primary and

organotypic culture systems are able to capture interactions between differentiating neurons, glial cells, and other cell types, development of functionally competent neurons and the formation of complex structures [25–32].

Stem cell differentiation models are rapidly emerging as valuable tools for assessing developmental neurotoxicology in human cells. In early brain development, pluripotent stem cells are differentiated to multipotent neural progenitor cells (NPCs) which can then differentiate into neurons, oligodendrocytes, and/or astrocytes [33]. Proliferating NPCs can be maintained indefinitely *in vitro* and introduction of controlled differentiation conditions yields a range of specific neuronal subtypes [34]. Previous characterization of *in vitro* stem cell differentiation has generated rich datasets on the essential processes underlying brain development and pathology that can be occurring during long-term neuronal differentiation *in vitro* [35].

Stem cell-based models have already been widely applied for neurodevelopmental toxicity testing, to evaluate toxicant perturbation of differentiation status, regional identity, neurite outgrowth, migration, synaptogenesis, and gene expression [16, 30, 31, 36–44]. In order to interpret and integrate results from these promising toxicological models, it is important to define the biological domains captured in the model and their relevance to specific *in vivo* contexts [45].

1.3 Anchoring neuronal differentiation *in vitro* to *in vivo* development

Translation of the results of these *in vitro* models for risk assessment requires a clear delineation of precisely how the developmental processes captured in each model reflect *in vivo* developmental processes. Tremendous progress has been made in high-resolution characterization of gene expression throughout human brain development and transcriptomic data are publicly available for specific brain structures as they form and mature through time [46, 47]. These rich *in vivo* datasets allow us to anchor *in vitro* toxicological screening models to *in vivo* biology. Furthermore, tools like the Gene Ontology (GO) Database and GO-Elite [48] can facilitate comparisons across *in vitro* and *in vivo* data by allowing quantitative characterization of biological processes captured in genomic data. Comparing *in vitro* models to early brain development *in vivo* can reveal developmental processes that define windows of susceptibility to toxicant perturbation that are captured *in vitro*. In the research presented here, we anchor the neurodevelopmental processes that occur *in vitro* to *in vivo* processes identified in publicly available datasets.

In our work, we characterize pathway dynamics throughout neuronal differentiation of a human neural progenitor cell (hNPC) line derived from NIH approved H9 embryonic stem cells [49, 50] that provides a particularly promising, scalable and reproducible model for high throughput and high content neurodevelopmental toxicity screening. In the absence of fibroblast growth factor, these hNPCs consistently undergo a dramatic morphological and functional transition to become mature, excitable neurons engaged in complex neuronal networks [51]. This cell line has already been proposed as a tool for development of high throughput toxicological screening methods [36, 37]. We have previously applied this model to study differential effects of chlorpyrifos and arsenic on proliferating and differentiating cells [13] and to evaluate neurotoxicity mechanisms of several other toxicants (unpublished).

Others have applied it to document toxicant-induced perturbation of differentiation (Table 1).

We used a quantitative pathway-based approach previously employed in our lab [52] to define pathway dynamics throughout time in differentiation conditions *in vitro*. We next compared global gene expression patterns during differentiation *in vitro* with gene expression in a publicly available dataset [47] that captures gene expression in neocortical brain regions during embryonic and early fetal stages of development. This comparison allowed us to evaluate the ability of the *in vitro* model to capture well-defined, conserved processes that drive development throughout the brain, including proliferation, migration, differentiation, synaptogenesis, apoptosis and neurotransmission [1]. This analysis demonstrates that a readily reproducible *in vitro* toxicological model reflects many of the developmental pathways and processes that are active in human brains at the earliest stages of development. Our baseline characterization of developmental dynamics captured in this *in vitro* model will be an essential tool for designing and interpreting results of future neurotoxicity testing in the context of the specific pathways and processes that are active at the time of treatment.

2. Materials and methods

2.1 Cell Preparation

Commercially available human neural progenitor cells (hNPCs) derived from NIH approved H9 stem cell line with normal female karyotype were obtained (ArunA Biomedical, Athens, GA, now available as ENStem-A hNPCs from Millipore, Billerica, MA) and expanded 8–10 passages in ENStem-A™ neural expansion medium (Millipore, Billerica, MA). For long-term differentiation experiments, hNPC culture protocols were modified from protocols developed by Aruna Biomedical (Athens, GA). At passages 8–10, cells were plated at a density of 200,000 cells/ml in 35mm dishes coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) diluted 1:200 in DMEM/F-12. Cells were allowed to attach and proliferate for 24 hours; then the medium was replaced with Hyclone neuronal differentiation medium (Thermo Fisher Scientific, Waltham, MA) in the absence of growth factor to initiate differentiation. Cells were maintained in differentiation conditions for up to 21 days, with half of the medium refreshed every two to three days and whole medium refreshed weekly.

2.2 Live/Dead Cell Imaging

In order to characterize morphology and cell viability through time in culture, differentiating cultures were incubated 15 minutes at 37° C with Calcein AM, Propidium Iodide, and Hoechst 33342, to specifically stain live cells, dead cells, and nuclei, respectively. Cells were visualized with a fluorescent microscope at 200x magnification. Images were processed, and color channels were combined using ImageJ software [53]. Images from days 0, 1, 3, 7, 14 and 21 are presented in this paper as representative samples.

2.3 Western Blotting

Western blotting was used to quantify changes in expression of key protein markers of differentiation status and function, over time. Protein samples were harvested in cell lysis

buffer (Cell Signaling Technology, Danvers, MA) at 0, 1, 3, 5, 7, 10, 14, and 21 days following initiation of differentiation conditions. Protein was isolated, sonicated, and quantified by protein assay (BioRad Laboratories, Hercules, CA) according to kit protocol. Western samples were prepared by appropriately diluting samples to equal protein concentrations and addition of sample loading buffer, and reducing agent (Life Technologies, Carlsbad, CA). Protein samples were loaded with at least 10 µg/well in a 4–12% bis-tris gel and Western gels were run at 200V. Protein was transferred to poly-vinyl difluoride membrane in chilled transfer buffer (BioRad Laboratories, Hercules, CA). Following transfer, membranes were washed in Tris-buffered Saline (TBS) and blocked in 5% milk in TBS with Tween (TTBS). Membranes were washed in TTBS and incubated at 4° C overnight with primary antibodies diluted in the primary dilution buffer.

Primary antibodies include β -tubulin III, MAP-2, Nestin, alpha synuclein, PCNA, and β -actin, and secondary antibodies include goat anti mouse (BD Biosciences, Franklin Lakes, NJ) and anti rabbit (BD Biosciences, Franklin Lakes, NJ). Membranes were washed in TTBS and incubated with secondary antibody diluted in 5% Milk in TTBS. Following secondary antibody incubation, membranes were then washed then incubated with ECL buffer (GE Healthcare, Chicago, IL).

Film was exposed to ECL-reactive membranes and developed. Intensity of western bands were quantified with ImageJ software and normalized to β -actin. Fold change expression intensity at each timepoint over average expression across time was reported reflecting results of three independent experiments.

A linear mixed effect model was used to evaluate change in protein expression over time, where day was a fixed effect and replicated samples as random effect variable.

2.4 Immunofluorescence

Plates were fixed in 4% paraformaldehyde for 30 minutes at 0, 1, 3, 5, 7, 10, 14, and 21 days following initiation of differentiation and stored in PBS at 4° C. Once all time points were collected, plates were washed 3 times in PBS and incubated with blocking buffer (5% Goat serum in PBS with Tween) to permeabilize cell membranes for 1 hour and then incubated overnight with primary antibodies diluted in antibody dilution buffer (5% BSA in PBS with Tween). Plates were washed the next day in PBS then incubated in Alexa-flour tagged secondary antibodies in antibody dilution buffer. Plates were then washed in PBS with Hoechst in the final wash to counterstain nuclei. Labeled cells were visualized under a fluorescent microscope at 200 and 400x magnification and images were processed and compiled using ImageJ software.

2.5 Microarray Processing

RNA from differentiating hNPC plates was harvested in Trizol 0, 1, 3, 7, 14, and 21 days following initiation of differentiation. RNA was then isolated using an RNA isolation kit (Ambion/mirVana; Thermo Fisher Scientific, Waltham, MA) according to kit protocol and samples were further purified with a cleanup kit (Qiagen, Hilden, Germany) according to kit protocol. Purified RNA samples were then hybridized to Affymetrix GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) to measure global gene expression in each

sample. Expression data reflects replicate samples from 3 independent cell preparations, and the Bioconductor Limma package was utilized to normalize and annotate obtained expression data [54].

2.6 Human *in vivo* Microarray Data from the Allen Brain Institute

We used an *in vivo* dataset of temporal gene expression in the developing neocortex [47]. This dataset includes global gene expression across a range of specific brain regions from four human subjects (N=4, all male) at gestational weeks 5.7, 6, 8, and 9. These were categorized into two distinct periods: the embryonic period (which includes weeks 5.7 and 6) and the early fetal period (which includes weeks 8 and 9). Kang, Kawasaki [47] characterized *in vivo* human brain gene expression with Affymetrix Human Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). For comparison with *in vitro* gene expression, *in vivo* gene expression data was pooled across all neocortical brain regions from samples within each period.

2.7 Gene Expression Analysis Pipeline

Overall similarity in gene expression between individual biological replicates across timepoints *in vitro* was compared through a principal component analysis (PCA). The PCA was conducted and visualized in the Affymetrix Expression Console software version 1.4.1.6 (Life Technologies, Carlsbad, CA) using signal (CHP) data following RMA normalization of probe level (CEL) data. Microarray CEL files were analyzed using R [55] with the library packages, limma [54], oligo [56], NBclust [57], annotate [58], and pd.hugene.1.0.st.v1 [59]. Probe features were normalized with the rma function in Affy package [60]. The getNetAffx [56] and Affycore tools [61] were used to annotate probe features. Fitting of the expression values for replicates was performed by the lmFit function. After normalizing and annotating gene expression, a criterion of FDR < 0.01 was applied to identify significantly changed genes across time. These criteria for significant changes were selected to maximize the number of genes with high biological importance included in the analysis. Significantly changed genes were then grouped according to general expression dynamics through time using *K*-means clustering [62] in R. Specifically, expression of each significantly changed gene at each timepoint was divided by the average expression of that gene across all timepoints. The NbClust package was used to determine the optimal cluster count for the kmeans function. This clustering method identifies the optimal number of clusters and assigns genes to clusters that most closely reflect their temporal expression patterns. The fold change in log2 expression for each gene at each timepoint was then used at the basis for *K*-means clustering. *In-vivo* and *in-vitro* data sets were analyzed separately.

2.8 Quantification of Pathway Dynamics *In vitro*

Significantly changed genes were identified and clustered according to expression dynamics as described above. In order to identify GO biological processes enriched among each cluster of significantly changed genes, we used GO Elite gene ontology software [48]. Enrichment analysis was performed with 2000 permutations of ORA using a permutation p-value cutoff of 0.05, and a minimum number of 3 genes changed for term enrichment. Terms with ID counts greater than 10000 were excluded. Enriched GO terms were ranked by *Z*-score.

$$Z - \text{score} = (r - n*(R/N))/((n*(R/N))(1 - (R/N))(1 - ((n - 1)/(N - 1))))^{1/2} \quad (1)$$

where N is the total number of genes measured, R is the total number of genes significantly changed, n is the total number of genes in each specific GO term, and r is the number of genes significantly changed within this specific GO term. The Z -score therefore represents the ratio of number of genes predicted to be randomly changed within a given GO terms and the number of genes actually changed within that term divided by the standard deviation of the observed number of genes in a hypergeometric distribution. Terms with a Z -score below 1.96 were excluded. In addition to identifying enriched GO biological processes, GO Elite quantifies the temporal dynamics of expression within each enriched term by averaging the relative log2 fold change expression of all the genes within each cluster that contribute to enrichment of a given GO term at each timepoint. Complete quantitative enrichment results for each cluster are available, ranked by Z -score in supplemental tables. To identify pathways and processes that are dominant within the distinct phases of differentiation described by each cluster, we categorized terms significantly enriched among each of the clusters by general themes. We selected a subset of GO terms that represent dominant themes in each cluster and plotted the average log2 fold change in expression intensity for each GO term through time to illustrate temporal trends.

2.9 Comparison of Global Gene Expression *In vivo* and *In vitro*

For comparisons with the *in vitro* model, genes differentially expressed between the embryonic and early fetal periods *in vivo* were identified using an FDR < 0.01. Gene expression in all neocortical brain regions in both individuals within each time period was averaged to compare differences in gene expression between the two periods. The set of genes differentially expressed across the two *in vivo* developmental stages were compared to the set of genes significantly changed through time *in vitro*. In order to compare pathways that may be active in early human brain tissues *in vivo* and differentiating hNPCs *in vitro*, we used GO Elite software to identify GO biological processes enriched among genes significantly changed across time in both *in vivo* and *in vitro* and among genes changed uniquely only *in vivo* or only *in vitro*.

2.10 Comparison of *In vitro* and *In vivo* Gene Expression in Targeted Pathways of Interest

In addition to the untargeted analysis described above, we characterized gene expression dynamics of targeted biological pathways and specific gene targets of interest. Pathways of interest as targets for developmentally neurotoxic chemicals were identified based on evidence from the published literature. We queried the Gene Ontology database (May 2014), for a list of human genes associated with each GO biological pathway of interest. We then compared gene expression patterns and relative expression intensity *in vitro* and *in vivo* for targeted pathways of interest for neurodevelopment (Fig 1). Heatmaps were utilized to visualize changes in the log2 gene expression at each timepoint *in vitro* and across individuals at early stages of brain development *in vivo* for each GO term of interest. In each heatmap, gene expression *in vitro* and *in vivo* for a specific pathway of interest was presented in rank order (y-axis) from highest to lowest mean gene expression through time

based on *in vitro* data (x-axis). The color scale depicts log2 gene expression levels where green represents low expression levels and white indicates high expression levels.

2.11 *In vitro* Gene Expression of Specific Gene Targets of Interest

To evaluate the presence of several specific genes of interest, we analyzed the lists of genes highly expressed among differentiating cultures. Genes were ranked in order of mean expression intensity at each time point *in vivo* and *in vitro*. Genes were considered expressed when their expression was above 50th percentile of expression among all other genes at each time point.

3. Results

3.1 Morphological Changes and Longterm Viability

Human neural progenitor cells were cultured in differentiation conditions for up to 21 days and evaluated for viability and morphological features. Under differentiation conditions, *in vitro* hNPC cultures increasingly displayed characteristics of differentiated neural networks. Fluorescent images illustrate formation of three-dimensional neural networks and extension of neurite outgrowths. Live-dead cell staining through time indicates that some cell death occurs as cultures undergo dramatic remodeling to form dense neuronal foci. However, a strong population of viable cells was consistently maintained (Fig 2).

3.2 Protein Expression

Progression of cells through neuronal differentiation was further illustrated by the dynamic changes in protein expression over time in culture. Western blots demonstrate that expression of the neuronal differentiation markers beta-tubulin III and microtubule-associated protein 2 (MAP2) increased significantly ($p<0.05$) through time (Fig 3). We also observed a significant increase in alpha-synuclein ($p<0.05$) over time, indicating an increase in the presence of synapses, which are a necessary condition for formation of functional neural networks.

The neural progenitor cell marker nestin also increased over time ($p<0.05$), indicating a growing population of cells that continued to maintain multipotency even as differentiation progressed (Fig 3). Continual PCNA protein expression through time indicated the presence of proliferative cells for at least 21 days following initiation of differentiation. Simultaneously, total protein content (Fig 4) significantly increased through time ($p<0.05$), likely due to a combination of continued cell proliferation and increasing mass of each differentiating and maturing neuron as it accumulates additional complex structures.

Immunofluorescent imaging of beta-tubulin III further demonstrated the progression of differentiation in culture. The images offer a qualitative confirmation of the increase in beta-tubulin III expression and demonstrate dramatic morphological development, with the extension of neurite outgrowths and formation of neural networks over time in differentiating conditions (Fig 5).

3.3 Global gene expression and pathway dynamics *in vitro*

Developmental pathway dynamics captured in the *in vitro* hNPC culture were characterized based on temporal changes in global gene expression (Fig 1a). Principal component analysis was used to compare gene expression trends of biological replicates at each *in vitro* timepoint (Fig 6). This analysis illustrates differences in gene expression trends across time. Biological replicates within each timepoint cluster together, indicating consistency in overall expression trends across replicates.

Over 21 days in differentiating conditions, 9482 genes significantly changed ($\text{FDR} < 0.01$) through time. We used *K*-means clustering to group significantly changed genes with similar expression dynamics. This revealed three major clusters of genes with distinct temporal trends (Fig 7).

A quantitative pathway analysis using GO Elite software identified GO biological processes significantly enriched ($Z\text{-score} > 1.96$) among each of the three *in vitro* clusters. Temporal dynamics for these GO biological processes are summarized according to the average gene expression of the genes contributing to the enrichment of each term (Fig 8; complete lists of enriched GO terms, accession numbers and genes contributing to enrichment of each term are available in Supplemental Table 1).

Significantly changed genes in Cluster 1 ($n=2922$) typically peaked in expression around differentiation day 3 and then decrease over time. Genes in this cluster are largely enriched for GO terms associated with cell cycle, stem cell proliferation, regulation of gene expression, and differentiation and development. Genes in Cluster 2 ($n=1560$) generally decreased in expression through time. Among these genes, significantly enriched GO terms were primarily associated with fate commitment, cell death, metabolism and transport, DNA replication, and response to stimulus. Genes in Cluster 3 ($n=5000$) generally increased in expression through time. GO terms significantly enriched among genes in Cluster 3, included those related to morphological development, axon guidance, synapse formation, migration, and signal transduction.

3.4 Comparison of GO biological processes enriched among genes significantly changed through time *in vivo* and *in vitro*

We used a publicly available dataset of human gene expression in 4 early samples of the developing neocortex [47] as an *in vivo* reference point for comparison with *in vitro* hNPCs. The dataset included global gene expression across a range of specific brain regions from 4 individuals estimated to be at postcoital weeks 5.7, 6, 8, and 9. Samples staged at weeks 5.7 and 6 are expected to capture the early stages of brain development in the embryonic period, while samples staged at weeks 8 and 9 are expected to capture the early fetal period, during which brain tissue becomes increasingly differentiated. Comparison of gene expression in brains at each of the two periods (embryonic vs. early fetal) revealed 7264 genes that were differentially expressed ($\text{FDR} < 0.01$). This set of genes differentially expressed between the embryonic and early fetal periods is the basis for comparisons with *in vitro* expression dynamics. Of 7342 genes identified *in vivo*, only 7264 genes were included for further analysis as 34 of them did not have a gene ID.

We compared the set of genes significantly changed through time *in vitro* with the set of genes differentially expressed between embryonic and early fetal stages of development *in vivo* (Fig 1b). There were 3792 genes changed both *in vivo* and *in vitro*, 3472 genes changed only *in vivo*, and 5690 genes changed only *in vitro* (Table 2; gene lists available in Supplemental Table 2). Using these gene lists, we identified GO biological processes enriched among genes that were significantly changed in both systems as well as processes enriched among genes changed only *in vivo* or only *in vitro* (Table 2; a full list of enriched terms and the genes associated with each term is available in Supplementary Table 1).

GO term enrichment among genes significantly changed both *in vivo* and *in vitro*.—Among 3792 genes significantly changed across time both *in vivo* and *in vitro*, pathway analysis reveals enrichment for terms related to general developmental processes including signal transduction and development and morphogenesis as well as specific processes such as neuronal differentiation, neurotransmission, and development (Table 2).

GO term enrichment among genes significantly changed *in vitro* only.—Among 5690 genes that were changed through time only *in vitro*, there was enrichment for terms relating to conserved developmental pathways, cell signaling pathways and stress and apoptosis pathways (Table 2). There were no brain-specific GO terms enriched among genes changed *in vitro* that were not also enriched *in vivo*. Enrichment for several non-brain developmental processes (e.g. ‘lung morphogenesis’ and ‘glomerulus development’) may reflect expression of signaling programs that regulate a diverse range of developmental processes.

GO term enrichment among genes significantly changed *in vivo* only.—Among 3472 genes that are changed across time only *in vivo*, significantly enriched GO terms reflected many of the same general processes reflected in GO terms enriched among genes significantly changed both *in vivo* and *in vitro*, including differentiation and morphogenesis pathways (Table 2). *In vivo*-specific enrichment of more specific neuronal subtype differentiation (e.g. ‘cerebral cortex neuron differentiation’) and neurotransmission may indicate that aspects of these specific processes are not fully captured in the *in vitro* model.

3.5 Comparison of gene expression in targeted pathways of interest for neurodevelopment *in vivo* and *in vitro*

Biological processes of interest for developmental neurotoxicology were selected based on key processes identified in seminal review papers as targets for neurotoxicity [1, 63]. Processes of interest for neurotoxicity include neural proliferation, differentiation, neuronal subtype specification, synaptic transmission, epigenetic regulation, response to hormones, oxidative stress, and inflammation. Heatmaps (Fig 9 and Supplemental Fig 1 – 7) provide a qualitative comparison of log₂ gene expression intensity for genes associated with each GO biological process that were significantly changed across time *in vitro* with expression intensity in four *in vivo* samples from early stages of brain development. Expression intensity of genes associated with some targeted pathways of interest were strikingly similar between *in vitro* and *in vivo* samples. For example, there appears to be substantial concordance between *in vitro* and *in vivo* expression intensity for genes associated with

proliferation, forebrain neuron differentiation, histone modification, and DNA methylation (Fig 9). These heatmaps highlight both similarities and differences between *in vivo* and *in vitro* gene expression patterns and illustrate temporal expression dynamics *in vitro* for specific genes and processes that may be altered by neurotoxic exposures.

3.6 Expression of specific gene targets *in vitro*

Specific gene targets can be important mediators of developmental neurotoxicity. We therefore probed lists of genes expressed *in vitro* to determine the presence or absence of gene targets of interest. We probed the lists of genes expressed (above the top 50th percentile of expression) among differentiating cultures and genes increasing through time in culture to confirm expression of several specific genes. These include neurotransmitter receptors and regulators, hormones and nuclear receptors, growth factors, metabolic enzymes and other genes that may mediate pathways of developmental neurotoxicity. Differentiating hNPCs in our model express both glutamate and GABA receptor subunits. Glutamate receptor subunits expressed in this culture, include AMPA (e.g. Gria1), NMDA (e.g. Grina, Narg2), and Kainate (e.g. Grik1, Grik2, Grik4) related subunits. GABA receptor subunits expressed in this culture include metabotropic (e.g. gabbr1) and nicotinic (e.g. CHRNA5, CHRNA2, CHRNA7) subunits. In addition, a range of hormonal regulators and nuclear receptors (e.g. THRA, RARA, RXRa, AR, LHCGR) and metabolic enzymes (e.g. cyp26A1, cyp1B1, hsd11b11, hsd17b12, hsd17b4) that could play an important role in mediating toxicity are all expressed *in vitro*. Identification of the specific targets expressed in this culture can inform design of assays that target appropriate pathways that are present in the model. For example, domoic acid is a strong agonist for kainite receptors and a partial AMPA agonist [64]. Expression of several relevant receptor targets in the hNPC culture suggest that this culture could be a suitable model for evaluating the excitotoxicity of developmental neurotoxicants like domoic acid.

4. Discussion

There is a pressing need to develop and validate *in vitro* models for rapid developmental neurotoxicity screening that captures sensitive pathways and processes relevant to *in vivo* conditions [21]. This work provides a temporal roadmap of *in vitro* neuronal differentiation in the hNPC model and is the first to anchor gene expression patterns *in vitro* to gene expression during sensitive windows of *in vivo* human development. Our analysis provides a foundation for defining potential toxicological applications of a readily reproducible *in vitro* model of neuronal differentiation.

4.1 Temporal dynamics of differentiating hNPCs

Under differentiation conditions, these hNPC cultures increasingly express classic markers of differentiated neurons [65] and basic patterns of morphology are consistent with known patterns of morphology described in previous neuronal differentiation models, including murine neural progenitor cells [11], rat/mouse micromass primary cultures [66, 67], and neuronal differentiation of induced pluripotent stem cells [38]. The three clusters of temporal gene expression patterns identified in our untargeted analysis illustrate a dynamic sequence of distinct gene expression phases driving differentiation and development *in vitro*.

Clearly defining the timing of these processes and quantifying overall pathway changes in this hNPC system creates a solid foundation from which to design *in vitro* toxicology studies that target phases of susceptibility for particular processes of interest.

4.2 Anchoring Gene Expression *In vitro* to Gene Expression *In vivo*

The human developmental brain tissue samples we used as an *in vivo* reference in this analysis represent two early stages of brain development that are susceptible to toxicant perturbation. Because human brain samples from these early stages of development are rare, we have a very limited *in vivo* dataset to work with. Inherent uncertainty around the precise gestational age of each of the four individual samples further limits our ability to interpret temporal gene expression dynamics *in vivo*. However, the available data do allow us to summarize broad trends in gene expression that may occur in neocortical tissue between the embryonic and early fetal periods. The embryonic period includes gestational weeks 5.7 and 6 and captures a phase during which the neocortical brain regions are in early stages of neurogenesis [1, 47]. This makes gene expression in brain tissue samples from the embryonic period a valuable point of reference for undifferentiated proliferating neural progenitor cells present during early stages of neurogenesis *in vitro*. The early fetal period starts at gestational weeks 8 and 9 and captures later stages of neurogenesis when cells in many brain regions are actively progressing through migration and differentiation [1, 47]. The increasingly differentiated tissue in this period provides an *in vivo* reference point for more differentiated cultures *in vitro*. The estimated gestational ages of our *in vivo* reference samples are therefore consistent with known windows of susceptibility to toxicant perturbation. Our comparisons of genes significantly changed through time *in vivo* and *in vitro* indicate substantial overlap in the processes captured during the early stages of neural differentiation and brain development *in vivo* and differentiating neural progenitor cells in the *in vitro* model. Evaluation of GO biological processes that are enriched among genes that are significantly changed in each system defines similarities and differences between processes that are active *in vivo* and *in vitro*. Targeted analysis of specific biological processes and gene targets of interest for neurotoxicology further highlights similarities and differences between *in vitro* and *in vivo* expression and provides a temporal roadmap for each of these processes in the differentiating *in vitro* system.

4.3 Evaluation of hNPC model success in capturing fundamental processes of brain development

Foundational papers of developmental neurotoxicity describe the roles of proliferation, migration, differentiation, synaptogenesis, gliogenesis, apoptosis and neurotransmission in shaping brain development and mediating toxicity [1, 2]. Toxicant perturbation of any of these basic developmental processes during critical periods of sensitivity can lead to adverse outcomes *in vivo*. We used our observations of viability, morphology, protein expression, gene expression dynamics, and comparisons of gene expression *in vitro* and *in vivo* to evaluate the success of this *in vitro* hNPC model in capturing these fundamental processes of early brain development (Table 3). Processes enriched among genes changed through time *in vivo* but not *in vitro* point to opportunities to improve the model's ability to capture later *in vivo* processes. Recently developed methods for culturing neuronal tissue illustrate opportunities to build increasingly complex models that more fully capture *in vivo*-like

conditions. The most sophisticated stem cell-based models are able to recapitulate specific brain structures *in vitro*. For example, several emerging stem cell models methods successfully capture the complex ‘inside-out’ nature of cortical development, in which neurons migrate outward upon differentiation and establish clear cortical layers [68–70]. Some labs have even produced cerebral “organoids” that contain both neurons and glial cells and recapitulate aspects of cortical development *in vitro* [71]. Other recent research has produced automated methods to systematically test the effects of varying combinations of small molecule signals on neuronal fate determination *in vitro* [72]. These new approaches offer exciting opportunities to capture a wide range of processes associated with specific regional identities and diverse neural subtypes during specific life-stages. For applications in toxicology, the complexity of a model system has to be balanced with consistency and reproducibility for high throughput and high content screening. For this reason, these more complex models need to be supplemented with more easily reproducible and scalable models. Models that can be differentiated from commercially available neural progenitor cell line while retaining an organotypic context, can provide context-specific toxicological screening data in a readily reproducible method.

4.4 Conserved developmental signals

In addition to these specialized processes of brain development, pathway analysis of temporal dynamics *in vitro* demonstrates that this model also captures many of the highly conserved hormonal cues and developmental signaling pathways that regulate a diverse range of early developmental processes [73]. GO terms enriched among genes significantly changed through time *in vitro* include terms associated with conserved signal transduction pathways (e.g. ‘regulation of canonical Wnt signaling pathway’ and ‘regulation of cellular response to growth factor stimulus’ enriched in Cluster 3) and general differentiation and morphogenesis processes (e.g. ‘anatomical structure development’ and ‘extracellular matrix organization’ enriched in Cluster 3) that are ubiquitous in development. The ubiquity of these signals in development is apparent in our analysis through enrichment for unrelated developmental processes that are directed by the same signaling programs (e.g. ‘face morphogenesis’ or ‘positive regulation of cardiac muscle cell differentiation’).

4.5 Applying the model for developmental neurotoxicity testing

Each of the basic developmental processes captured in this model may be sensitive to perturbation by a diverse range of toxicants during critical periods of sensitivity. However, the sensitivity of each of these processes is likely to vary depending on the timing of exposure. In the field of developmental neurotoxicity, there is increasing appreciation for the importance of life course evaluation and identification of specific windows of susceptibility. For example, we have previously demonstrated that arsenic and chlorpyrifos have differential effects in this hNPC model under proliferating and differentiating exposure conditions [13]. Furthermore, recent work has illustrated crucial species differences in gene expression dynamics of neuronal cultures through time [74]. Such findings illustrate the importance of designing toxicology studies that identify and target specific developmental contexts *in vitro*. We therefore performed this in-depth characterization of developmental dynamics to identify windows of human susceptibility for specific pathways of interest in this *in vitro* model. The resulting temporal roadmap for this *in vitro* model (illustrated in

Figures 3, 8 and 9) is a tool to help identify optimal exposure windows for future evaluation of toxicant effects on specific pathways of interest. Figure 8 showed the temporal dynamics of GO biological processes in the *in vitro* system and illustrates, along with Figure 9 (and supplemental Figures 1 to 7), that our *in vitro* system reflects the embryonic and early fetal period identified for human brain week 5.7 to week 9.

Given the wide range of mechanisms that can result in developmental neurotoxicity, it is of particular importance that this culture system is able to capture aspects of many of these processes in a single *in vitro* model. However, one model alone cannot provide a complete picture. It must be applied as part of a panel of models [17] reflecting a range of developmental contexts and genetic backgrounds that, together, can more comprehensively anticipate adverse perturbation of pathways and processes throughout brain development.

In this analysis, we showed two types of comparisons between *in vivo* and *in vitro* dynamics. We reported overall gene changes, showed all significantly changed genes, and indicated genes in common and unique to *in vivo* and *in vitro* systems. We see significant changes in genes associated with neurite outgrowth and synapse formation both *in vivo* and *in vitro*, but this analysis on its own cannot tell us what these pathway dynamics mean for development of functional synapses *in vitro*. Further exploration of specific gene changes in each pathway and evaluation of functional endpoints will be necessary to determine how overall gene expression dynamics result in functional changes in each pathway. Gene expression dynamics identified here can be used to prioritize further testing and characterization of protein, functional endpoints and specific toxicological targets *in vitro*. Finally, we would like to acknowledge that the human neural progenitor cells that we used are of female origin whereas the *in vivo* data are from developing brain of male origin; therefore, potential sex-specific differences during neurodevelopment may be present.

Anchoring dynamic *in vitro* models to *in vivo* developmental contexts is an important first step in identifying opportunities to apply these models in neurotoxicity testing. This type of baseline analysis provides an important foundation for designing toxicity studies that evaluate effects of chemicals *in vitro* accounting for the specific biological context of a specific *in vitro* model at a specific time. With this baseline characterization, models like this can be used to determine how perturbation of specific developmental pathways and endpoints *in vitro* contribute to adverse outcomes of toxicants *in vivo* [21]. These analyses provide a systems-based temporal roadmap providing insight for pathway dynamics *in vitro*. This will provide important context for experimental design and interpretation of future studies that apply this *in vitro* model to predict developmental neurotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Differentiation is characterized for human neuro progenitor cells
- This transcriptomic signature can identify normal and perturbed differentiation
- Genes changed in vivo and in vitro showed concordance
- Neurotransmission gene pathways may not be fully captured in the in vitro model

Figure 1. Overview of gene expression analysis.

Methods for A) quantification of temporal pathway dynamics in differentiating hNPCs *in vitro*, B) unsupervised comparison of processes occurring *in vitro* with processes occurring in early stages of brain development *in vivo*, and C) comparison of *in vivo* and *in vitro* expression for targeted pathways of interest.

Figure 2. Longterm viability and morphology of differentiating hNPCs.

Qualitative evaluation of cell viability was performed with live/dead cell imaging. Images from 0, 1, 3, 7, 14, and 21 days following initiation of differentiation are presented as representative samples. Cells were incubated for 15 minutes at 37° C with Calcien AM, Propidium Iodide, and Hoechst 33342 dyes to stain live cells (green), dead cells (red), and total nuclei (blue), respectively. Cells were visualized with a fluorescent microscope at 200x magnification. Morphological imaging reveals that over time in differentiating conditions, hNPCs form dense neural clusters.

Figure 3. Protein expression in differentiating hNPC cultures through time.

Protein was harvested from differentiating hNPCs and expression of specific markers was evaluated by western blotting, with equal amounts of protein loaded in each sample. Data is normalized to Actin expression and presented as fold change expression intensity at each timepoint over expression average across time and reflects results of three independent experiments. Error bars indicate standard error. β -tubulin III, MAP2, α -synuclein, and nestin expression all increase significantly over time ($p < 0.05$). Representative Western blots for each protein marker are included for reference.

Figure 4. Accumulation of protein content through time in differentiating hNPCs.

Protein was harvested from differentiating hNPCs through time and quantified by Bradford protein assay. Data represents 3 independent experiments. Error bars indicate standard error of the mean. Protein content increases significantly across time ($p < 0.05$).

Figure 5. Morphological development of differentiating hNPCs.

Differentiating hNPCs were fixed and β -tubulin III expression was visualized with a fluorescent tag (green). Nuclei are counterstained with Hoechst 33342 (blue). Cells were visualized with a fluorescent microscope at 400x magnification. The increased expression of beta-tubulin III expression and morphological development indicates a growing population of differentiating neurons.

Figure 6. Principal component analysis (PCA) of gene expression across *in vitro* timepoints. PCA plot was generated using signal (CHP) data following RMA normalization of probe level (CEL) data. Signal data from three biological replicate microarrays for six time points are shown as colored boxes representing days following initiation of differentiation (0–21).

Figure 7. K-means clustering of genes significantly changed through time *in vitro*.

Unsupervised *K*-Means clustering identified 3 dominant patterns of expression among genes significantly changed through time in differentiating hNPCs. The fold change in log2 expression intensity of each gene was calculated at each timepoint by dividing log2 expression at that timepoint by average expression of that gene across all timepoints. *K*-means clustering was used to group genes according to the resulting fold change in log2 expression at each timepoint for each gene. The heatmap summarizes temporal expression trends for genes in each cluster by showing the average fold change in Log2 gene expression across all genes at that timepoint.

Figure 8. Temporal dynamics of GO biological processes enriched among genes changed under differentiation conditions *in vitro*.

GO biological processes significantly enriched ($Z > 1.96$) among each of these clusters were identified by GO Elite pathway enrichment analysis and grouped by general themes.

Examples of enriched GO terms in each cluster are presented here. Temporal dynamics of enriched GO terms are illustrated by plotting the average log2 fold change in expression intensity of genes contributing to enrichment of each GO term through time.

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F. DNA Methylation

G. Chromatin remodeling

Fig 9. Example heatmaps comparing *in vitro* vs. *in vivo* gene expression for genes involved in proliferation, differentiation, fate commitment, and epigenetic changes.

Heatmaps show log₂ gene expression for individual genes at each timepoint in *in vitro* and *in vivo* for biological processes related to (A) hNPC proliferation, (B) neuronal fate commitment, (C) cortical neuron differentiation, (D) forebrain neuron differentiation, (E) histone modification, (F) DNA methylation, and (G) chromatin remodeling. Left panels show *in vitro* expression across time. Right panels show *in vivo* expression across four individuals from early stages of brain development. Heatmaps only include genes associated with each GO biological process that were changed through time *in vitro*.

Table 1.

Example of toxicants evaluated using *in vitro* human neural progenitor cells for potential developmental neurotoxicity.

Compounds	Evaluated hNPC conditions	Exposure duration	References
Chlorpyrifos	Proliferation, Differentiation	24hr	Kim, H.Y., Wegner, S.H., Van Ness, K.P., Park, J.J., Pacheco, S.E., Workman, T., Hong, S., Griffith, W.C., and Faustman, E.M. (2016) Differential epigenetic effects of chlorpyrifos and arsenic in proliferating and differentiating human neural progenitor cells. <i>Reprod Toxicol</i> . 65:p.212–223.
Chlorpyrifos	Proliferation, Differentiation	24hr	Wu, X., Yang, X., Majumder, A., Swienburg, R., Goodfellow, F.T., Bartlett, M.G., and Stice, S.L. (2017) From the cover: astrocytes are protective against chlorpyrifos developmental neurotoxicity in human pluripotent stem cell-derived astrocyte neuron cocultures. <i>Toxicol Sci</i> . 157(2):410–420.
Arsenic	Proliferation, Differentiation	24hr	Kim, H.Y., Wegner, S.H., Van Ness, K.P., Park, J.J., Pacheco, S.E., Workman, T., Hong, S., Griffith, W.C., and Faustman, E.M. (2016) Differential epigenetic effects of chlorpyrifos and arsenic in proliferating and differentiating human neural progenitor cells. <i>Reprod Toxicol</i> . 65:p.212–223.
Silver Nanoparticles	Proliferation, Differentiation	24hr	Park, J.J., Hong, S., Workman, T., Griffith, W.C., and Faustman, E.M. (2017) Effects of silver nanoparticles with various coatings and sizes on proliferating and differentiating human neural progenitor cell (hNPCs) <i>in vitro</i> . Ph.D. Dissertation. University of Washington.
Bisphenol A, Acetaminophen, Bisindolylmaleimide I	Differentiation	up to 14 days	Wu, X., Majumder, A., Webb, R., and Stice, S.L. (2016) High content imaging quantification of multiple <i>in vitro</i> human neurogenesis events after neurotoxin exposure. <i>BMC Pharmacol Toxicol</i> . 17(1):62
Polychlorinated Bisphenyls	Differentiation	7 days	Fritsche, E., Cline, J.E., Nguyen, N., Scalan, T.S., and Abel, J. (2005) Polychlorinated bisphenyls disturb differentiation of normal human neuroprogenitor cells: clue for involvement of thyroid hormone receptors. 113(7):871–876.
Azacytidine, Trichostatin	Differentiation	48hr	Majumder, A., Dhara, S.K., Swetenburg, R., Mithani, M., Cao, K., Medrzycki, M., Fan, Y., and Stice, S.L. (2013) Inhibition of DNA methyltransferases and histone deacetylases induces astrocytic differentiation of neural progenitors. <i>Stem Cell Res</i> . 11(1): 574–586.

Note: Fritsche et al. (2005) used human neural progenitor cells but in neurosphere format.

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Table 3

Fundamental processes of brain development captured *in vitro*.

Process that Drive Brain Development	Examples of Toxicants Previously Shown to Perturb These Processes in the Literature	Evidence for Each Process in Culture?	Lines of Evidence		
			Protein Expression & Morphological Evidence	In Vitro Gene Expression Dynamics GO biological processes enriched among g genes changed through time in vitro	Concordance with In Vivo Expression GO biological processes enriched among genes significantly changed both in vitro and in vivo
Proliferation & Progenitor cell maintenance	valproic acid [Go et al., 2012] ethanol [Gohlke et al., 2008, Guerri et al., 1998]	yes	PCNA, Nestin, increasing protein content	cell cycle process; mitotic cell cycle; regulation of cell cycle; neural precursor cell proliferation	neuronal stem cell maintenance; negative regulation of stem cell proliferation; re-entry into mitotic cell cycle
Migration	deltamethrin [Kumar et al., 2013], ethanol [de la Monte et al., 2009]	yes	morphological observations	cilium movement; neuron migration; locomotory behavior; negative regulation of cell adhesion	regulation of mononuclear cell migration; locomotion; negative regulation of cell motility
Early Neuronal Differentiation	Retinoic acid, cyclopamine [Zimmer et al., 2011].	yes	Beta-tubulin III, MAP2, alpha-synuclein	neural crest cell differentiation; regulation of neurogenesis	positive regulation of stem cell differentiation; epithelial cell differentiation
Neurite Outgrowth and Synaptogenesis	Anesthetics [De Roo et al., 2009], cadmium [Pak et al., 2014]	yes	alpha-synuclein, morphological observations	cell projection organization; neuron projection guidance; synapse organization; dendrite morphogenesis	synapse organization; neuron projection guidance; positive regulation of cell projection organization
Neuronal Subtype Specification and Regionalization	methylmercury [Zimmer et al., 2011], bisphenol A [Huang et al., 2017]	yes (ate-specific)	(no data)	cerebral cortex development	dopaminergic neuron differentiation; dopamine biosynthetic process; forebrain anterior/posterior pattern specification
Neurotransmission	pesticides [Wilson et al., 2014; Torres-Altoro et al., 2011; Shafer et al., 2008], metals [Sal-q et al., 2012; Minami et al., 2001], perchlorate [Gilbert and Sui, 2008], air pollution [Davis et al., 2013]	yes (signal-specific)	(no data)	neurotransmitter receptor metabolic process; neuron-neuron synaptic transmission; neurotransmitter uptake; negative regulation of neurotransmitter secretion; regulation of synaptic transmission	positive regulation of synaptic transmission, dopaminergic; L-glutamate transport; cerebellar Purkinje cell layer development;
Gliogenesis & Myelination	ethanol [Guizzetti et al., 2014] diazinon [Pizzurro et al., 2014], rotenone and paraquat [Rathnam et al., 2012]	no	(no data)	N/A	N/A
Apoptosis	dioxin [Xu et al., 2013], trichloroethylene [Salama et al., 2018], trichloroethane [Kajita et al., 2019].	yes	morphological observations	regulation of neuron death	regulation of neuron apoptotic process; negative regulation of neuron death; fibroblast apoptotic process

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