Introduction
This project is part of the programmed research initiated by the National Institutes of Health, the US Food and Drug Administration and the Defense Advanced Research Projects Agency to develop human-on-a-chip tools to assess the safety and efficacy of countermeasures to biological and chemical terrorism and warfare. This challenge requires not only the development of the in vitro model mimicking the human organs, as described here for the central nervous system (CNS), but also novel bioengineering and quality assurance techniques. The latter will require adapting tools from Good Cell Culture Practice [1] and validation. Here, we describe the adaptation of a three-dimensional (3D) organotypic CNS model for human induced pluripotent stem cells (iPSCs).

There is a critical lack of knowledge when it comes to toxicity of drugs and other xenobiotic chemicals on the developing brain. Only very few substances have so far been confirmed to result in developmental neurotoxicity (DNT) [2], while neurodevelopmental disorders such as autism and attention-deficit hyperactivity disorder are being diagnosed at ever-increasing rates [3]. A critical constraint for routine DNT assessment of drugs and chemicals is the high cost of testing, approximately $1.4 million per substance for the current regulatory test strategy according to the DNT guidelines [4,5]. But there are also scientific concerns regarding the relevance of these studies for human health effects. Testing is typically
performed at high doses that are not relevant for human exposure scenarios, since human exposure often occurs at low doses over prolonged time periods. Nonhuman animal testing also does not reflect inter-individual differences among the human population. Moreover, the relevance of behavioral and histological data from rodents for human health has been questioned [6]. Consequently, current guidelines often do not provide sufficient information to facilitate regulatory decision-making.

**Modeling the complexity of the central nervous system in vitro**

3D models are far superior for recapitulating the complex directional growth and connections that underlie neurodevelopment [7]. A two-dimensional model constrains cellular morphology, preventing complex synaptic interactions. In contrast, a 3D model enables neurons and astrocytes to assume a more natural shape and extend processes to synapses and neighboring cells.

Given the importance of cell-to-cell interactions in the brain, our laboratories have begun characterizing a 3D rat primary aggregating brain cell culture model, granted by the US Food and Drug Administration (#U01FD004230), in order to map pathways of DNT. We and others have used a variety of techniques to show the presence of all relevant cell types in these cultures [8-12]. Moreover, synaptogenesis and myelination can be observed. Consequently the model has the potential for detecting chemicals interfering with these processes; for example, by blocking the release of neurotrophic factors or interfering with cell adhesion. We are currently using emerging technologies to study neurotoxicity in these cultures [9,10]. For example, we have identified metabolic profiles that can distinguish compounds with different target organ toxicity (brain, kidney and liver). The DNT consensus process identified our rat aggregating culture model among the most representative models for DNT studies [13-15].

To increase the usefulness of the model, we are adapting our model for studying cells of human origin. The in vitro data from the rat and the human model can then be compared to identify possible species differences to better predict the potential for human toxicity. Such an innovative approach is expected to provide more precise information for human risk assessment, and regulatory decision-making, than the current extrapolations and predictions based on animal models. In the field of drug development, about 92% of substances fail during clinical trials due to effects in humans that were not identified in preclinical animal tests [16]. The use of human cell models is therefore crucial and has the advantage of eliminating interspecies confounds.

The use of stem cells has the potential to create new humanized models for toxicity testing. Stem cells have the capacity for self-renewal and can differentiate into all cell types of the CNS, such as neurons, astrocytes and oligodendrocytes [17]. The main sources of generating stem cells with the potential to differentiate into neural lineages are pluripotent embryonic stem cells [18-20], human umbilical cord blood-derived neural stem cells [21], multipotent somatic stem cells derived from bone marrow [22,23] or other tissues including the CNS [24] as well as iPSCs [25].

Generating stem cells from any one source will have advantages and disadvantages. In this project we chose to use iPSCs, which overcome many limitations of other sources; for example, ethical issues, limited accessibility, and restricted genetic backgrounds. In addition, iPSCs seem to be more stable, with a higher neuronal differentiation efficiency than, for example, somatic stem cells [26,27]. However, generating iPSCs is challenging. First, the percentage of reprogrammed cells after induction of pluripotency genes is often low. Moreover, many differentiation protocols still require further optimization because the procedure to obtain mature neurons and especially glial cells is very time consuming and the reproducibility and efficiency of differentiation can be low. In this project we are evaluating different protocols and culturing techniques to obtain a reproducible model with increased efficiency of targeted differentiation. To establish a human aggregating model, we will combine astrocytes and neurons derived from iPSCs [28,29]. Methods have been described for differentiation of iPSCs to astrocytes and different types of neurons [28,30-32]. iPSC lines from different individuals will provide a testing model with the ability to predict substance sensitivity in different genetic backgrounds. Moreover, the development of neurons and glia from iPSCs is believed to recapitulate many stages of brain development in utero [32]. Each stage of neurodevelopment is unique and displays different sensitivities to diverse xenobiotics. A DNT in vitro model should cover many of these stages.

**Induced pluripotent stem cell differentiation**

We have optimized protocols for differentiating human iPSCs into neural progenitor cells (NPCs) and neuronal and astroglia lineages (Figure 1). First, the generation of NPCs involves a stepwise neural differentiation protocol through embryoid body formation as previously described [25,33]. iPSCs are detached after treatment with collagenase (1 mg/ml) for 1 hour and plated onto nontreated polystyrene plates in human embryonic stem cell medium in the absence of fibroblast growth factor but in the presence of heparin with the medium exchanged daily. After 4 days in this medium the cells are switched to human NPC medium (DMEM/F12, neurobasal, heparin, N2), exchanged every 2 days. After 2 weeks in suspension cultures, embryoid bodies are collected...
and mechanically dissociated into smaller cell clusters. These cells are plated onto poly-l-ornithine and laminin-coated plates and passaged mechanically as adherent cultures (Figure 1a) in human NPC expanding medium (KnockOut DMEM/F12, Glutamax, endothelial growth factor and basic fibroblast growth factor), neural supplement and penicillin–streptomycin. To determine the purity of the culture, we stain cultures for neural progenitor markers nestin and Sox-2 (Figure 1b).

In the second protocol, for neuronal differentiation of NPCs, we plate early-passage NPCs on poly-l-ornithine and laminin-coated plates, with neuronal culture medium (neurobasal medium supplemented with 2 mM l-glutamine, B27) for 1 week to generate immature neurons (Figure 1b). Immature neurons are then cultured in neuronal medium supplemented with brain-derived neurotrophic factor (10 ng/ml) and glial cell-derived neurotrophic factor (10 ng/ml) for 4 to 8 weeks to obtain mature neurons.

In the final protocol, NPCs at a later passage are induced to differentiate into astrocytes by withdrawal of fibroblast growth factor-2 and addition of ciliary neurotrophic factor and 10% fetal bovine serum for 2 to 4 weeks.

Figure 1. Generation of neurons from induced pluripotent stem cells. (a) Phase contrast images showing undifferentiated induced pluripotent stem cells (iPSCs; left panel); neural progenitor cells (NPCs) 7 days after neural induction (middle panel) and after passage 1 of the NPCs (right panel). (b) Expression of NPC markers Sox-2 and nestin co-stained with 4′,6-diamidino-2-phenylindole (DAPI; left and middle panels) show maintenance of undifferentiated NPCs over several passages. Cells expressing neural markers (microtubule-associated protein-2, MAP2) and synaptic (synapsin1), co-stained with DAPI, 21 days after differentiation (right panel). (c) Expression of neuronal marker (MAP2) plus co-expression of markers for specific cortical layers (Tbr1, Brn2, and Ctip2), co-stained with DAPI, 4 weeks after differentiation.
To determine the purity of cultures, we immunostain differentiated cells for glial fibrillary acidic protein and S100β (astrocytic markers), DCX and microtubule-associated protein-2ab (neuronal markers), O4 and glutathione transferase π (oligodendrocytic markers). Extensive dendritic growth is observed by staining for microtubule-associated protein-2, and synapses are indicated with antibodies against synapsin-1 (Figure 1b). Neural progenitors can differentiate to neurons representative of cortical layers (Figure 1c) and GABAergic and glutameric neurons as indicated by the expression of markers specific for cortical layers (Tbr1, Brn2, and Ctip2), glutamate decarboxylase, and the vesicular glutamate transporter, respectively (data not shown).

Preparation of human three-dimensional neural cell cultures

The 3D human neural model is prepared using similar techniques to those established for our 3D rat primary aggregating brain cell cultures [34]. Three approaches have so far been tested. First, cells are differentiated from NPC rosette aggregates (Figure 2a) directly in 3D (Figure 2d), inducing the differentiation using differentiation media as described above for 4 weeks. For the second approach, cells from human NPC adherent cultures (Figure 2b) are detached and plated in uncoated six-well plates with a density of 2×10^6 human NPCs/well (Figure 2c) for 4 weeks. In the final approach, human NPCs (Figure 3a) are differentiated two-dimensionally...
for 4 weeks (Figure 3b). After 4 weeks of differentiation, cells are detached and cultured in a 3D suspension as previously described with a density of 2×10^6 cells/well (Figure 3c). Cultures are maintained at 37°C in an atmosphere of 10% CO_2 during constant gyratory movement.

Similar to our rat brain aggregates, the 3D human neuronal model is characterized by (RT-)PCR and immunocytochemistry for selected markers that have been found to be involved in neuronal and glial differentiation in primary rat cultures [35,36] (Figures 2 and 3). In addition, neurotransmitter receptor activity will be assessed by measuring intracellular messengers including calcium and cAMP. Our results so far have shown higher expression of various neuronal differentiation markers in the aggregated cultures obtained from single cells than from the aggregates obtained directly from rosettes (Figure 2). Moreover, aggregates from neurons differentiated for 4 weeks in two dimensions show increased expression of different neuronal markers after the 3D formation, suggesting that the model continues to mature in 3D (Figure 3).

**Genetic sensitivity to xenobiotics**

A limitation to traditional animal model-based chemical and drug testing has been the inability to address the influence of (epi)genetic background and medication history on sensitivity. To investigate the impact of different genotypes of neurons derived from iPSCs on chemical sensitivity, we will generate iPSCs from patients with neurodevelopmental disorders such as Rett syndrome, tuberous sclerosis complex and Down syndrome. We chose these disorders as a point of entry because of their potential sensitivities to substances that induce oxidative stress [37]. Greater than 90% of the cases of Rett syndrome are due to methyl CpG binding protein 2 and mouse models of Rett syndrome have shown increased oxidative damage in different tissues [38]. Tuberous sclerosis complex is an autosomal dominant neurodevelopmental disorder caused by mutations in the TSC1 and TSC2 genes. In the developing child, intractable epilepsy, cognitive impairment, and autism have been reported [39]. Down syndrome is due to trisomy in chromosome 21 and is associated with impaired neurodevelopmental maturation and early neurodegeneration. Several genes are expressed at higher levels due to the trisomy, which affects reactive oxygen species including superoxide dismutase.

iPSCs derived from fibroblasts of individuals with these disorders, as well as healthy individuals, will undergo neural differentiation and exposure to chemicals with predicted neurodevelopmental toxicity (Table 1). First, we will analyze the cellular response to the toxicants in the 3D neural model derived from healthy iPSC donors by measuring oxidative damage and morphological and functional endpoints such as immunohistochemistry, gene expression and calcium flux. Second, we will determine whether iPSC-derived neurons and astrocytes from patients with neurodevelopmental disorders exhibit

![Figure 3. Three-dimensional aggregates formed from cultures pre-differentiated in two dimensions for 4 weeks.](image-url)
generate viable models of the human developing brain. Better understanding of how to apply this technology to after chemical exposure. Many challenges remain to be may vary, which will complicate the evaluation of effects and produce reactive oxygen species. Differentiation and culturing physiology and pathology. However, several challenges represent a versatile tool for more complex testing plat- forms and strategies as well as research into CNS selection of DNT chemicals, presented in Table 1.

Table 1. Potential developmental neurotoxicity chemicals to be tested in aggregating brain cell cultures

| Chemical       | Exposure       | Toxic effects and/or main mechanisms of toxicity                                                                 |
|----------------|----------------|---------------------------------------------------------------------------------------------------------------|
| Aspartame      | Food additive  | Excitotoxicity mainly through activation of the NMDA-R, reduction of acetylcholine esterase activity           |
| Bisphenol A    | Plastic additive| Endocrine disrupter at very low doses, can suppress cell proliferation, can induce apoptotic cell death       |
| Cadmium chloride| Environmental contaminant, smoking | Causes oxidative stress and affects genes involved in cell cycle regulation                                   |
| Carbaryl       | Pesticide      | Affects neurite outgrowth, inhibits nitric oxide synthesis and inhibits acetylcholine esterase                |
| Chlordane      | Pesticide      | Inhibits acetylcholine esterase, induces damage to RNA and DNA synthesis, oxidative stress, astroglial proliferation and cell differentiation |
| Lamotrigine    | Anti-convulsant drug | Interferes with the voltage gated sodium channels and has shown teratogenic effects in some studies         |
| Lead chloride  | Environmental contaminant | Associated with numerous adverse effects in the central nervous system, including destruction of the blood–brain barrier, loss of neurons, gliosis and oxidative stress |
| Lindane        | Pesticide      | Inhibits acetylcholine esterase, noradrenaline uptake, GABA neurotransmission and blocks glycine receptors |
| Maneb          | Pesticide      | Inhibits GABA synthesis, causes loss of dopaminergic and GABAergic neurons, decreases ATP levels and causes oxidative stress |
| Trichloroethylene| Environmental contaminant | Associated with adverse effects in the central nervous system, induces loss of dopaminergic neurons and oxidative stress |
| Valproic acid  | Antiepileptic drug | Recognized as a teratogenic compound, modifies the release of GABA                                             |

Dysfunctional developmental processes as predicted by disease-relevant mutations. Finally, we will examine whether these genetic mutations increase sensitivity to a selection of DNT chemicals, presented in Table 1.

Development of such a 3D human brain model will represent a versatile tool for more complex testing platforms and strategies as well as research into CNS physiology and pathology. However, several challenges have to be considered. Differentiation and culturing protocols need to be optimized to generate reproducible models consisting of the same cell types. In this project, three different protocols are evaluated to generate the most promising 3D neuronal model. Differentiation of the NPCs as monolayer cultures before culturing in 3D will allow for better control over the cell fate of the progenitors upon aggregation. However, this approach may not be able to capture the early neurodevelopmental window, a sensitive stage of development that is of high importance. Similar challenges will probably be faced for the disease model. One cannot guarantee that iPSC-derived cells from a disease-permissive genetic background will retain relevant epigenetic modifications or exhibit clear developmental phenotypes. Several epigenetic modifications in the donor tissue might be lost or modified by reprogramming of the cells. In addition, the differentiation efficiency of cells from different donors may vary, which will complicate the evaluation of effects after chemical exposure. Many challenges remain to be overcome, but as the iPSC field matures we will gain a better understanding of how to apply this technology to generate viable models of the human developing brain.

Abbreviations

CNS, central nervous system; 3D, three-dimensional; DMEM, Dulbecco’s modified Eagle’s medium; DNT, developmental neurotoxicity; iPSC, induced pluripotent stem cell; NPC, neural progenitor cell; PCR, polymerase chain reaction; RT, reverse transcriptase.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

All authors contributed equally.

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