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Review Article

Exploring Environmental Neurotoxicity Assessment Using Human Stem Cell-Derived Models

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Abstract

Neurotoxicity is increasingly recognized as a critical factor impacting long-term health, with growing evidence linking it to both neurodevelopmental and neurodegenerative diseases. Pesticides, widely used in agriculture and industry, have emerged as significant contributors to neurotoxic risk, given their capacity to disrupt key neurodevelopmental processes at low exposure levels. As conventional animal models present limitations in interspecies translation, human-derived neuron-based in vitro screening strategies are urgently needed to assess potential toxicants accurately. Human-induced pluripotent stem cells (hiPSCs) offer an innovative and scalable source for human-specific neuronal models that complement traditional animal-based approaches and support the development of predictive assays for neurotoxicity. Recent various stem cell models, including 2D cultures, 3D organoids, and microfluidic systems, are now available, advancing predictive neurotoxicology by simulating key aspects of human neural development and function. With the integration of High-Throughput (HT) and High-Content (HC) screening methodologies, these hiPSC-based systems enable efficient, large-scale evaluation of chemical effects on neural cells, enhancing our ability to detect early biomarkers of neurotoxic effects. Identifying early biomarkers of neurotoxic is essential to developing therapeutic interventions before irreversible damage occurs. This is particularly crucial in the context of developmental neurotoxicity, where early exposure to toxicants can have lifelong consequences. This review specifically presents an in-depth overview of the current progress in hiPSC-derived neural models and their applications in neurotoxicity testing, with a specific focus on their utility in assessing pesticide-induced neurotoxicity. Emphasizing future research priorities, we highlight the potential of these models to transform predictive toxicology, offering more human-relevant assessments and advancing the field toward a more precise evaluation of environmental neurotoxicants.

More Information

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Introduction

Human brain development involves complex processes susceptible to chemical disruption, which can lead to irreversible nervous system impairments and elevate risks of neurodevelopmental and neurodegenerative diseases [1,2]. Research indicates that the immature nervous system, particularly during fetal and early postnatal periods when the Blood-Brain Barrier (BBB) is still developing, is especially vulnerable to chemical exposure [3,4]. Evaluating neurotoxic chemicals thus requires attention to exposure timing, as critical developmental windows show heightened sensitivity to toxicants and are associated with long-lasting neurological effects [5]. There is strong evidence linking prenatal exposure to neurotoxicants with structural and functional deficits later in life, often mediated by epigenetic changes affecting brain development [6,7].

The exposome, introduced by Chris Wild in 2005, encapsulates all environmental exposures across an individual's lifetime, advancing risk assessment and understanding gene-environment interactions [8,9]. This concept, combined with epigenetics, allows researchers to explore how environmental factors modulate gene expression through mechanisms such as DNA methylation, illuminating the pathways by which cumulative exposures during critical developmental periods can affect brain health [10].

As a response to these insights, human induced pluripotent stem cell (hiPSC)-derived neuronal cultures have emerged as practical alternatives to traditional animal models. hiPSCs replicate human physiology more closely, providing better predictions of neurotoxic effects [11-13]. Available in two-dimensional (2D), three-dimensional (3D), and microfluidic formats, hiPSC models allow human-relevant neurotoxicity assessments and hold promise for predictive screening to identify early biomarkers of neurological disorders [14,15].

This review discusses iPSC-based brain models, particularly their applications in neurotoxicity assessment, with an emphasis on pesticide effects. By examining these models, we aim to highlight their potential in identifying early biomarkers and in elucidating mechanisms underlying pesticide-induced neurotoxicity.

The relevance of stem cell models

Over the years, neurotoxicity research has utilized a range of *in vitro* models, each offering unique insights but also presenting limitations. Traditional models like immortalized cell lines and primary cultures have been instrumental in focusing on specific neural cell types for targeted neurotoxicology studies, but are inherently restricted by genetic modifications and short lifespans, leading to compromised physiological relevance [13,16].

Stem cells have since emerged as a versatile platform in toxicology. Offering self-renewing, pluripotent, or multipotent sources, stem cells allow for differentiation into diverse neural lineages, thus enhancing model relevance for human applications [17]. Depending on origin, these cells are categorized into Adult Stem Cells (ASCs), Fetal Stem Cells (FSCs), Embryonic Stem Cells (ESCs), and induced Pluripotent Stem Cells (iPSCs). However, ESCs, derived from early-stage embryos, offer high differentiation potential but face significant ethical constraints [18,19]. ASCs and FSCs, while ethically viable, are limited to specific lineages and lack the full pluripotency seen in ESCs [20,21].

Induced pluripotent stem cells (iPSCs) are somatic cells reprogrammed to a pluripotent state, an achievement first realized in 2006 with murine cells by Yamanaka's team [22] and later applied to human cells [23]. The process involves introducing key transcription factors—such as c-Myc, Klf4, Sox2, and Oct3/4—using retroviral vectors, enabling iPSCs to differentiate into various cell types. With robust self-renewal and pluripotency, human iPSCs (hiPSCs) provide significant potential as human-relevant models for neurotoxicity and developmental neurotoxicity (DNT) screening [24].

Induced Pluripotent Stem Cell (iPSC) models: Advancing neurotoxicity assessment

Induced pluripotent stem cells (iPSCs) offer a promising alternative for neurotoxicity assessment, particularly given the ethical and practical limitations associated with human embryonic stem cells (hESCs). Unlike immortalized cell lines and primary cultures, iPSCs can differentiate into a range of neural cell types, including neurons and glial cells. These cells enable comprehensive DNT assessment through endpoints such as proliferation, differentiation, apoptosis, migration, neurite outgrowth, and network activity [25,26].

hiPSC-derived models address the ethical concern surrounding hESC and the limitations of interspecies extrapolation by more closely reflecting human neurodevelopment, providing ethically sound data that align with the 3R principles (Replacement, Reduction, and Refinement) [27,28]. Furthermore, these models enable the examination of gene-environment interactions, assessing how environmental factors, combined with genetic predispositions, influence neural function and contribute to pathological phenotypes [29].

For neurotoxicity assessment, iPSC models can mimic long-term exposure effects, elucidating mechanisms of diseases thought to originate from fetal exposure and revealing cumulative neurodevelopmental impacts of toxicants over time. Additionally, hiPSCs can be cryopreserved, generating diverse, consistent cell types—including neural stem cells, dopaminergic neurons, cortical glutamatergic neurons, oligodendrocytes, and astrocytes—facilitating scalability for neurotoxicity screening [30-32].

Advanced electrophysiological techniques, such as multi-well micro-electrode arrays (mwMEA) and calcium imaging, allow hiPSC-derived neurons to display mature network properties with spontaneous activity, enhanced by astrocyte co-culture [33-35]. These complex networks respond to neurotoxic compounds, providing data on excitatory and inhibitory balance crucial for toxicity studies [35-38].

Overall, hiPSC-based models hold great potential for advancing neurotoxicology, offering human-relevant data that surpasses traditional models in predicting adverse outcomes.

Platforms for neurotoxicity assessment using iPSC brain models

2D hiPSC neural differentiation models: Human-induced pluripotent stem cells (hiPSCs) serve as a versatile foundation for generating various neural cell types in 2D cultures, providing a practical framework for neurotoxicity studies. For example, hiPSCs from both healthy individuals and Parkinson's disease patients have been differentiated into dopaminergic neurons, establishing useful models for disease and neurotoxicity screening [39]. hiPSC-derived GABAergic interneurons, crucial for maintaining neural excitation balance, are generated through targeted transcription factors, facilitating the rapid differentiation into functional neurons [40,41]. Additionally, motor neuron

differentiation protocols enable the creation of spinal motor neurons that closely mimic human physiology, making them highly relevant for specific neurodegenerative studies [42,43].

Co-culturing hiPSCs-derived neurons with astrocytes enhances functional maturity in these neurons, with human astrocytes proving preferable over rodent cells to avoid interspecies variability [44]. Simplified differentiation protocols, such as those developed by Gunhanlar, et al. have refined this approach, allowing mature cortical neurons to form without astrocyte co-culture. This method consistently generated a 60:40 ratio of neurons and astrocytes, which result from a common forebrain neural progenitor and exhibit a similar neurodevelopmental process *in vivo*, thus providing streamlined model systems without sacrificing cellular complexity [45].

Although 2D hiPSC neural differentiation models are highly effective for rapid, HT neurotoxicity screening and allow detailed analysis of individual cell types, they inherently lack the structural complexity required to fully replicate multicellular interactions and neural network dynamics found *in vivo*. This limitation suggests the need for more advanced models that incorporate the intricate cellular interactions critical to accurate neurodevelopmental and neurotoxicological assessments.

Advancement from 2D to 3D models: The transition from 2D to 3D hiPSC cultures represents a significant advancement in creating *in vitro* models that more closely mimic the complex spatial organization of the human brain. While 2D models allow for efficient High-Throughput Screening (HTS), their limited dimensionality constrains the replication of *in vivo*-like cellular interactions and physiological relevance [16]. In contrast, 3D models, such as brain organoids and cortical spheroids, offer enhanced biological fidelity by fostering the development of cellular structures and interactions observed in the central nervous system [46]. These models not only support processes such as neural differentiation and synaptic connectivity but also allow for the organization of neural progenitors and mature neurons in a way that better resembles human brain architecture [46]. Therefore, 3D cellular organotypic models, including neural spheroids and organoids, have emerged as an alternative toxicity screening platform to traditional 2D *in vitro* and animal model approaches [47].

Through 3D induction, cell fate specification and neural network complexity are promoted, facilitating deeper insights into how chemicals may disrupt neurodevelopmental processes [48]. This shift toward three-dimensionality provides a promising bridge between *in vitro* studies and *in vivo* applicability, making 3D hiPSC models highly relevant for toxicology research that requires spatially complex cell interactions.

Efforts to further standardize and scale these models will be instrumental in expanding their accessibility for HT applications, thereby increasing the translational impact of *in vitro* neurotoxicity screening. Figure 1 illustrates both 2D and 3D models and their application in the field of neurotoxicity assessment.

3D hiPSC-derived brain organoids: 3D cerebral organoids derived from human pluripotent stem cells (hPSCs) represent an advanced model for studying human brain development and disease, as they partially replicate the early stages of fetal brain formation *in vitro* [49]. Organoids begin with embryoid body formation and progress

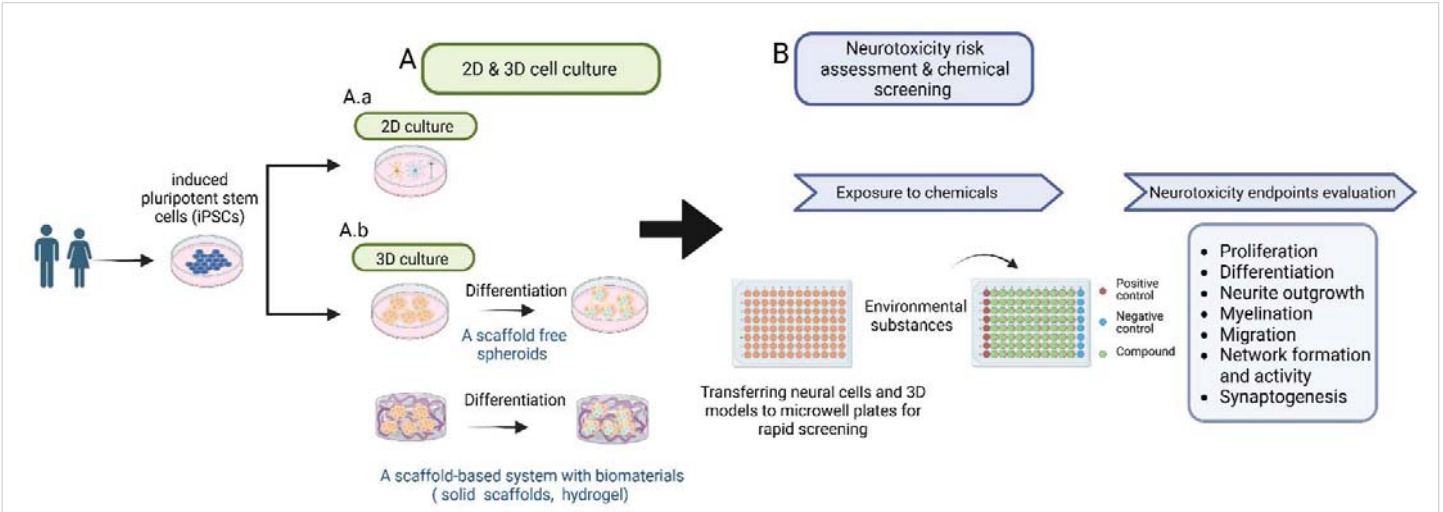

Figure 1: Comparison of 2D and 3D Cell Culture Models in Neurotoxicity Assessment. (A) Cell Culture Systems: (A.a) Traditional 2D cell culture on flat surfaces; (A.b) 3D cell culture systems, including scaffold-free methods (using ultra-low attachment or ECM-coated plates) and scaffold-based systems with rigid or soft biomaterials (e.g., hydrogels) that mimic ECM architecture. (B) Neurotoxicity Assessment and Chemical Screening: iPSC-derived 2D and 3D models are applied to screen and evaluate the neurotoxicity of chemicals using key neurotoxicity endpoints. (Figures created with BioRender.com).

through differentiation of the three germ layers (endoderm, mesoderm, and ectoderm), ultimately yielding structures that resemble specific brain regions, such as the forebrain, midbrain, and hindbrain, depending on differentiation cues provided [50]. Different approaches have led to region-specific organoid models, each tailored to mimic distinct aspects of brain development. For instance, forebrain organoids accurately model the ventricular zone structure with polarized SOX2-positive radial glial cells, essential for studying cortical layer formation and neuronal migration [51,52]. Midbrain organoids, replicate dopaminergic neuron populations, providing a platform for neurodegenerative disease studies [53], while cerebellar and hippocampal organoids mimic synaptic organization and connectivity relevant to neurodevelopmental disorders [54,55].

Recent advances include the development of "assembloids," or fused organoids, where multiple brain regions are combined to investigate cellular interactions and interneuron migration. Birey, et al. fused dorsal and ventral forebrain spheroids, simulating interneuron migration as observed *in vivo* [56]. Another notable advancement involves vascularized organoids, which enhance viability and cellular complexity by incorporating endothelial cells to simulate the Blood-Brain Barrier (BBB) and promote nutrient exchange, a crucial factor in long-term culture viability [57,58].

These 3D organoid models provide a versatile platform for neurotoxicity testing, allowing for dynamic interaction studies and exposure assessments that reflect more accurate human brain physiology than traditional 2D cultures.

Development of iPSC Blood-Brain Barrier (BBB) models: Effective neurotoxicity assessment requires models that account for a substance's ability to cross the Blood-Brain Barrier (BBB), a highly selective structure that controls molecular access to the Central Nervous System (CNS) [59]. The BBB comprises brain microvascular endothelial cells (BMECs), pericytes, and astrocytes, creating a barrier essential for maintaining CNS homeostasis. In recent years, several iPSC-based BBB models have emerged to replicate this structure, allowing detailed investigations into neurotoxic effects on BBB integrity and function [60,61].

Lippmann, et al. developed an iPSC-derived BMEC model that demonstrated strong barrier properties and efflux transporter activity, essential features of the *in vivo* BBB [62]. Co-culturing these BMECs with astrocytes has further enhanced their structural and functional integrity, achieving transendothelial electrical resistance (TEER) measurements close to *in vivo* values [62]. Advances have led to the development of BBB spheroids, in which BMECs, astrocytes, and pericytes self-assemble, enhancing permeability assessments in a 3D context (Urich, et al. 2013).

Organ-chip technology offers further advancements, with microfluidic devices that replicate the BBB's selective permeability. For instance, Wang, et al. created a BBB-on-chip system using iPSC-derived BMECs and rat astrocytes, successfully demonstrating a functional barrier with dynamic media flow [63]. More recent work by Vatine, et al. established an entirely human BBB-on-chip using iPSC-derived BMECs, astrocytes, and neurons, achieving physiologically relevant TEER values and enabling targeted drug administration studies [64].

These iPSC-based BBB models are promising tools for HT neurotoxicity screening, allowing researchers to simulate the effects of chemical exposure on BBB integrity and CNS homeostasis with human-relevant insights.

Development of iPSC vascularized organoids: The complexity of brain organoids has advanced significantly with efforts to incorporate vascularization, a key factor for mimicking *in vivo* brain environments and enhancing organoid longevity and function. The absence of vasculature in traditional organoid models restricts nutrient and oxygen diffusion, leading to hypoxic conditions and limiting growth beyond a certain size. Vascularized organoid models overcome these limitations by supporting endothelial cell differentiation and network formation, a critical step toward creating organoids with *in vivo*-like properties [57].

Various approaches to vascularization have been explored, including implanting organoids into immunodeficient mice, enabling host vasculature to penetrate and support the graft [65]. Mansour, et al. demonstrated this with hiPSC-derived cerebral organoids grafted into the mouse cortex, showing axonal projection and synaptic connectivity with the host tissue over time. Alternatively, vascular networks have been induced *in vitro* by embedding endothelial cells within organoids or by adding endothelial growth factors, as in the study by Shi, et al. [58], where co-culturing with human umbilical vein endothelial cells led to vascularized cortical organoids capable of supporting neurogenesis.

More advanced methods involve genetic engineering for vascularization, such as Cakir, et al. who used the ETV2 gene to induce early vascular development within cerebral organoids [66]. These vascularized organoids exhibit improved neuronal activity and maturation, closely resembling the structural and functional interactions found in human brain tissue. Such models hold great promise for studying neurovascular interactions in health and disease, providing a human-relevant platform for testing drug efficacy and neurotoxicity.

The new generation of risk assessment: iPSC microfluidic chip models: Microfluidic technology, initially developed to miniaturize lab processes for analytical applications, has evolved into a robust platform for HTS screening and toxicity testing. Characterized by micron-

scale channels, microfluidic systems integrate multiple experimental processes into a single device, enhancing reproducibility, reducing reagent use, and decreasing analysis time [67]. Applications in life sciences are particularly transformative, encompassing cell analysis, co-culture systems, and organ-on-chip (OoC) platforms for tissue-specific studies. OoCs, which emulate the architecture and function of human organs through 3D bioengineered constructs, improve cellular viability and offer a controlled environment for studying complex tissue interactions, such as those found in brain-on-chip models, essential for neurotoxicity studies [68].

The dynamic nature of microfluidic systems allows for real-time adjustments to culture conditions, enabling intricate toxicity and pharmacokinetic evaluations. For example, Sances, et al. demonstrated that an iPSC-derived spinal cord chip enhances neuronal activity and gene expression [69], while Liu, et al. created a 3D brain-on-chip model to assess pesticide toxicity on neuronal viability [70]. Further integration of iPSC-derived blood-BBB models has enabled studies on neurovascular interactions under continuous perfusion, enhancing BBB fidelity and drug testing relevance [71]. These findings allow simultaneous study of pharmacokinetics across interconnected tissues, promising a shift toward comprehensive toxicity assessment.

Despite these advances, challenges, including automation compatibility and scalability for HT needs remain. However, recent efforts to create automated, HT-compatible systems, such as the OrganoPlate, have demonstrated the potential of 3D microfluidic systems for screening environmental neurotoxicity at scale [72]. As microfluidic technology matures, its capacity for simulating human-specific responses in neurotoxicity studies positions it as a pivotal tool in regulatory and research applications, advancing both environmental health and personalized medicine. Table 1 summarizes the advantages and disadvantages of iPSCs-derived neural models.

Applicability of hiPSC-derived neural models for in vitro neurotoxicity screening

Given rising concerns about neurotoxicity resulting from chemical exposures during critical periods of human development, there is an urgent need for efficient *in vitro* neurotoxicity screening systems that leverage human-derived neurons, thereby reducing reliance on interspecies extrapolation. The human brain, characterized by extensive neuronal networks, complex synaptic connectivity, and a high diversity of gene expression, may be particularly vulnerable to environmental chemicals [73]. Although recently, approximately 200 chemicals have been identified as neurotoxicants impacting human development from tens of thousands of commercial compounds, many neurotoxic substances are likely still remain unidentified [74].

The current neurotoxicity assessment framework, which is heavily reliant on animal studies, is limited by ethical concerns, low throughput, and the inability to efficiently handle large chemical libraries. These limitations highlight an essential need for human-relevant, *in vitro* models that can be automated for HT screening applications [75-80]. Effective *in vitro* models should be capable of closely mimicking the complexity of the human brain, including forming functional neural networks composed of diverse neuronal subtypes, excitatory and inhibitory pathways, and key neuroglial cells, such as astrocytes, oligodendrocytes, and microglia, to accurately reflect human neurophysiology [81,82].

hiPSC-derived neuronal and glial models are particularly promising for neurotoxicity assessment, as they allow expansion and differentiation of neural cells from an undifferentiated state in culture, providing a scalable solution for tissue-specific toxicological studies [14,30,83]. hiPSC-derived models are capable of replicating key neurodevelopmental events, from Neural Progenitor Cell (NPC) proliferation to advanced neuronal and glial

Table 1: Advantages and disadvantages of iPSCs-derived neural models.

	2D	3D organoids and assemblies	BBB	Vascularized	Microfluidics
Advantages	Simple, cost-effective. Reproducible Highly scalable for rapid cellular, genetic, and signaling studies.	Mimic more *in vivo* complexity 3D cell-cell and cell-ECM interactions. Spatial organization.	Enables TEER (Transendothelial Electrical Resistance) measurements for barrier function evaluation. Allow permeability studies and multi-cellular co-culture.	Enhanced physiological functionality Presence of vascular networks. Improved cell-cell interaction and maturation (compared to BBB)	High sensitivity and precision. Efficient cell activity detection. Real-time cell monitoring. Multi-Condition Analysis. Analytical Integration (mass spectrometry, DNA sequencing, imaging and biosensors)
Disadvantages	Lacks 3D complexity Limited cell-cell and cell-extracellular interactions (ECM)	Higher cost (compared to 2D). Potential for tissue necrosis (lack of homogeneous distribution of oxygen and nutrients) Lacks vascularity Material variability used for scaffolding	Lacks full 3D vessel structure. Doesn't fully replicate selective transport of *in vivo* BBB.	Lack fully functional vascular systems. Require complex culture techniques Limited size Limited lifespan and stability	Complex device fabrication. Issues with sterilization and fluid dynamics. Challenges for High-Throughput (HT) applications. High cost (compared to the other models) Need for specialized expertise.

maturation. This ability offers quantifiable endpoints to assess the impacts of chemical exposure. Disruptions in these processes due to chemical exposure can be measured, providing valuable readouts for *in vitro* toxicity screening [84]. Table 2 summarizes iPSC-derived model applications in *in vitro* neurotoxicity studies, with a specific focus on pesticide exposure.

2D hiPSC-derived models and neurotoxicity assessment: The increasing prevalence of neuro-developmental disorders such as autism spectrum (ASD) disorders, Attention-deficit/hyperactivity disorder (ADHD), and neurodegenerative diseases like Parkinson's disease has prompted interest in evaluating the neurotoxic potential of environmental pollutants, particularly pesticides [85-94].

To date, some neurotoxicity studies have been published to test the applicability of iPSC-derived models as novel models to assess chemical toxicity *in vitro*. In a pivotal study, Pei, et al. evaluated the neurotoxicity of an 80-compound library, including neurotoxic pesticides such as chlorpyrifos, dieldrin, and aldicarb, on iPSC-derived models (NSCs, neurons, and astrocytes). This study underscores the model's ability to discern neural-specific toxicity patterns and highlights the high sensitivity of iPSC-derived neurons to neurotoxicants [73].

Similarly, Li, et al. conducted an HT screen of 84 compounds, including pesticides like rotenone, deltamethrin, and tebuconazole, using GFP-labeled iPSC-derived cortical and motor neurons to enable live, real-time imaging of neurite outgrowth [95]. Their findings demonstrated that pesticides such as parathion exhibited selective inhibition of neurite extension in cortical neurons, while rotenone reduced neurite length and number across both cortical and motor neurons in a dose-dependent manner [95]. By integrating HT assays with quantitative imaging analysis, this study illustrates the capacity of iPSC-derived models to not only screen compounds rapidly but also generate mechanistic insights into pesticide-induced neurotoxicity.

Further advancing the application of iPSC models, Di Consiglio, et al. examined Chlorpyrifos (CPF), an organophosphate pesticide linked to developmental neurotoxicity. The CPF exposure effects at concentrations ranging from 18.45 to 37.10 µM (IC20) over a 14-day period were evaluated using iPSC-derived NSCs undergoing differentiation toward neurons and astrocytes. Immunocytochemistry and High-Content Imaging (HCI) revealed that CPF exposure reduced the expression of synaptophysin, a pre-synaptic marker, and BDNF protein levels, both of which are critical for synapse formation and function [96]. This model demonstrated CPF's concentration-dependent neurotoxic effects, including reduced neurite outgrowth and synapse number, highlighting its utility for studying pesticide-induced neurotoxicity. This aligns with findings from several studies [38,72,97].

Mitochondrial activity and ATP levels are key indicators of cell viability, widely used in assessing neurotoxicity in iPSCs and NPCs. Kamata, et al. employed MTS 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-

Table 2: The application of *in vitro* iPSC-derived models for assessing pesticide-induced neurotoxicity.

iPSC model	Pesticides	Time of treatment	Concentration	Findings	References
hiPSC-derived neurons and astrocytes	Chlorpyrifos	14 days	37.1 µM, 21 µM, 0.37 µM	Alterations in synapse number, neurite outgrowth, BDNF levels, and neuron/astrocyte ratios.	[1]
hiPSC-NSCs	DDE, HCB, α-chlordane, oxychlordane, α-HCH, β-HCH, γ-HCH, Dieldrin	3, 14 and 28 days	concentrations comparable to Scandinavian human blood levels	Increased NSC proliferation, and reduced synapse numbers.	[2]
hiPSC-derived NSCs, neurons, and astrocytes	Aldicarb, captan, carbaryl, chlorpyrifos, deltamethrin, DDT, Dieldrin, Heptachlor, lindane, permethrin, rotenone, tebuconazole	2 days	Ranges from 0 µM to 1000 µM	Specific inhibition or upregulation was observed in key neural markers like PAX6, OTX2, and MAP2.	[3]
hiPSC-derived excitatory and inhibitory neurons	28 pesticides including rotenone and dimethoate	24h	0-20 µM	Disruptions in neural network formation and function.	[4]
Lineage-specific luciferase hiPSC lines	Deltamethrin, dieldrin, carbaryl, and rotenone	24h	10 and 100 µM	Cytotoxicity across iPSCs, NSCs, neurons, and astrocytes.	[5]
hiPSC-derived neurons and astrocytes.	Chlorpyrifos	3 days and 14 days.	0.49 to 500 µM.	Decrease of synaptophysin levels, BDNF increased, and reduced neurite outgrowth.	[6]
hiPSC-derived neurons and astrocytes.	Vinclozolin	3 days and 14 days	1 to 1340 µM	Increase of BDNF	[7]
3D hiPSC-derived multicellular brain spheroid.	Rotenone	24 h and 48 h	0 to 50 µM	Induction of dopaminergic toxicity at low concentrations, affecting astrocytes at high doses; dysregulated of neural gene expression	[8]
3D autism spectrum disorder (ASD) iPSC-derived brain organoid.	Chlorpyrifos (CPF)	24h at 4 weeks	100µM	CPF disrupted ASD metabolic biomarkers, neurotransmitters (glutamate/GABA), and dopamine level reduction.	[9]
3D hiPSC-derived neurospheres	Paraquat, rotenone	72h	Paraquat (1µM-1000µM) Rotenone (0.001 µM-1000µM)	Concentration-dependent cell death; neurite outgrowth and connectivity reduction.	[10]

2-(4-sulfophenyl)-2H-tetrazolium and ATP assays to screen pesticides and other developmental neurotoxicants across varying concentrations, identifying agents like captan, carbaryl, chlorpyrifos, dieldrin, deltamethrin, lindane, and rotenone as significant DNT-positives based on IC50 values [98]. The study further demonstrated that several pesticides interfere with neural differentiation markers (e.g., PAX6, OTX2, MAP2), a crucial pathway in neurodevelopment. CPF notably inhibited PAX6, emphasizing its potential to disrupt neuronal differentiation.

Moreover, assessing the effects of pesticide mixtures is critical, as environmental exposures often involve complex chemical cocktails. iPSC-derived neuronal/glial co-cultures and mathematical modeling have illuminated how chemicals with similar modes of action, such as those impacting BDNF levels, synergistically enhance neurotoxicity at human-relevant doses. Pistollato, et al. found that mixtures of BDNF-disrupting pesticides led to significant downregulation of synaptogenesis markers, particularly PSD95, suggesting synergistic impairment in neural connectivity [99]. Additionally, Davidsen, et al. highlighted the importance of synaptogenesis as a sensitive endpoint in the mixture exposures study, reinforcing iPSC-based models' utility in capturing the intricate impacts of pesticide interactions on neurodevelopment [100].

Moreover, Park, et al. employed a high-throughput ATP assay in iPSC-derived cortical neurons to investigate pesticide-induced changes in mitochondrial function, a critical indicator of neurotoxicity [101]. This study found that pesticides, including chlorpyrifos and deltamethrin, significantly disrupted mitochondrial activity, underscoring the relevance of metabolic assays in detecting neurotoxicity at various stages of neural differentiation.

To further expand the application of 2D hiPSC-derived models in neurotoxicity assessment, recent studies have demonstrated the efficacy of these systems in evaluating pesticide-induced neural toxicity. Ishibashi, et al. developed a robust *in vitro* platform using hiPSC-derived dopaminergic neurons to explore the neurotoxic effects of environmental pesticides. Their finding indicated selective vulnerability of dopaminergic neurons, relevant to Parkinson's disease [102]. This research highlights the model's sensitivity to specific pesticides, providing a basis for understanding dopaminergic neurodegeneration pathways triggered by pesticide exposure. Bartmann, et al. advanced this field by creating a human Neural Network Formation (hNNF) assay using co-cultured hiPSC-derived excitatory and inhibitory neurons with primary human astroglia [103]. This model was screened with a library of 28 chemicals, including various pesticides, to examine neurotoxicity on network parameters such as spike, burst, and synchronization characteristics. The results demonstrated the assay's utility in identifying neurotoxic compounds, establishing it as a valuable tool for environmental toxicology studies.

In parallel, Paul, et al. integrated epidemiological insights with a toxicity screen using hiPSC-derived dopaminergic neurons from Parkinson's disease patients [104]. Their study identified ten pesticides with significant neurotoxic effects, particularly on neurons implicated in Parkinson's disease pathology, offering mechanistic insights into how environmental factors might contribute to neurodegenerative processes.

Collectively, these studies validate the use of iPSC-derived 2D neural models for neurotoxicity screening, particularly in the evaluation of pesticide-induced toxicity. With integrated approaches such as High-Content imaging (HC), lineage-specific markers, and real-time monitoring, these models provide a comprehensive platform to assess the neurodevelopmental impact of pesticides and other environmental toxicants, contributing significantly to developmental neurotoxicity research and regulatory applications.

3D iPSC-derived organoids and neurotoxicity assessment: Brain-organoid technologies provide an advanced platform for studying human neural development, functionality, and associated disorders in *vitro*. Typically, neurotoxic substances are introduced into iPSC-derived brain organoids at defined time points, representing acute or prolonged *in utero* exposures. This approach allows researchers to observe key morphological, transcriptional, and functional changes, offering detailed insights into the impact of neurotoxic exposures during critical developmental windows [54,105,106]

While the Embryonic Stem cell Test (EST) remains a standard for assessing embryotoxicity [19], brain organoids derived from iPSCs enable a similar evaluation of neurotoxicants by simulating specific developmental stages and exposure scenarios.

Although no standardized DNT tests currently exist for iPSC-derived organoids, these models have already shown considerable potential in environmental neurotoxicant testing [82,107]. For instance, Pamies, et al. investigated the developmental toxicity of rotenone, a pesticide that inhibits mitochondrial Complex I, by exposing brain spheroids derived from iPSCs at varying stages of maturation [108]. The study found that early-stage BrainSpheres (2 weeks of differentiation) were significantly more vulnerable to rotenone-induced toxicity, showing increased Reactive Oxygen Species (ROS) levels and mitochondrial dysfunction. At low concentrations (1 μM), rotenone selectively affected dopaminergic neurons, while higher concentrations (25 μM) impacted astrocytes. Transcriptomic analyses highlighted activation of the p53 signaling pathway in early differentiation, with disruptions in synapse and calcium signaling pathways later stages.

Similarly, several studies have begun to explore how

environmental neurotoxicants interact with genetic susceptibilities in neurodevelopmental disorders. CPF, an organophosphate pesticide associated with ASD risk, was investigated in an iPSC-derived brain organoid model with a CHD8 heterozygous knockout, a mutation implicated in ASD [109]. In this study, it was presented that CPF exposure altered glutamate/GABA balance and reduced dopamine levels pointing to altered biomarkers linked with ASD and highlighting the complex gene-environment interactions that can be studied using iPSC-derived organoids.

In 2023, Lam, et al. integrated a 3D hydrogel tissue model with iPSC-derived neurons and primary astrocytes to evaluate neural activity changes in response to pesticide exposure such as dieldrin and CPF, facilitating detailed neural network activity analyses [110]. Additionally, a study by Mariani, et al. further demonstrates the application of 3D iPSC-derived organoids in pesticide neurotoxicity studies. Human iPSC-derived organoids were used to evaluate the neurotoxic effects of neonicotinoid pesticides, revealing disruptions in neural differentiation and synaptic formation. These findings demonstrated the potential of organoid models to capture specific impacts of pesticides on neurodevelopment, particularly in pathways associated with synaptogenesis and cellular maturation [111].

Despite their promising applications, 3D organoid models still face challenges, particularly regarding scalability for HTS. The structural complexity of these models makes their adaptation to HTS formats challenging. Kobolak, et al. addressed this by developing a scalable 3D neurosphere model in a 96-well plate format that includes neurons, astrocytes, and oligodendrocytes [112]. Their model was successfully evaluated to evaluate the cytotoxicity of pesticides, specifically paraquat and rotenone, demonstrating concentration-dependent cell death with IC50 values determined for each pesticide [112]. This approach provides a scalable and efficient framework for HT 3D neurotoxicity screening, showcasing the potential of iPSC-derived organoids in assessing pesticide-induced neurotoxicity.

Enhancing functionality for neurotoxicity screening, Sirenko, et al. developed a 3D neural platform with mature cortical neurons and astrocytes optimized for measuring calcium oscillations—a sensitive biomarker for neuronal activity and toxicity [113]. This screening assay facilitated the quantitative assessment of chemical effects on Ca^{2+} dynamics across a library of 84 compounds, including various pesticides [113].

Altogether, 3D iPSC-derived organoid models offer a compelling alternative to animal models in neurotoxicity research, capturing human-relevant molecular, cellular, and functional responses to neurotoxicants. They enable in-depth mechanistic studies of chemical interactions, including those modulated by genetic factors, positioning them as suitable tools for advancing personalized approaches in neurotoxicity and environmental health research.

iPSC-microfluidic brain organoids and neurotoxicity assessment: Microfluidic-based brain-on-chip systems represent a significant advancement in replicating the neurovascular environment and simulating the dynamic complexity of the human brain, improving the predictive value of *in vitro* neurotoxicity models. By incorporating 3D cultures, microfluidic channels, and endothelial cell-derived vascular compartments to replicate the BBB, these platforms offer enhanced capabilities for assessing how neurotoxicants, particularly pesticides, affect the brain at a mechanistic level [114,115]. This model's design, embedding brain tissue analogs such as astrocytes, pericytes, and microglia in an extracellular matrix, provides a more physiologically relevant environment for studying neurotoxicity.

Koo, et al. demonstrated the utility of a microfluidic platform by examining how organophosphate pesticides, including Diethyl Methylphosphonate (DEMP) and Dimethyl Methylphosphonate (DMMP), interact with BBB integrity and acetylcholinesterase (AChE) activity. Pesticides were tested over a 24-hour period, revealing that high concentrations of DEMP and DMMP ($>10^{-3}$ M) compromised cell viability and disrupted AChE activity [116]. Residual pesticide levels in the brain compartment indicated BBB permeability, confirming this system's capacity to model neurovascular dynamics accurately. This model also tested pesticides such as CPF, malathion, and parathion, demonstrating their capacity to penetrate the BBB, inhibit AChE, and induce cell toxicity within the brain tissue construct [117].

Recently, Amend, et al. evaluated the use of Polydimethylsiloxane (PDMS)-based organ-chip platforms for toxicokinetic assessment, particularly using the organophosphate pesticide parathion and nerve agent VX as case studies [118]. Neuronal and liver spheroids were used to monitor concentration changes in the system, revealing that high absorption of these compounds by PDMS material significantly affected bioavailability. This study emphasizes the importance of selecting appropriate materials or coatings to prevent compound loss, ensuring accurate toxicokinetic studies for organophosphates on organ-chip platforms [118].

These microfluidic brain-on-chip platforms exhibit promising correlations with *in vivo* toxicity data, capturing critical endpoints such as BBB penetration, AChE inhibition, and cell viability. They present a valuable tool for high-throughput neurotoxicity screening, especially in evaluating complex physiological responses to pesticides, potentially advancing personalized approaches to risk assessment in neurotoxicity research.

Opportunities and future aspects of the iPSC-based model for neurotoxicity assessment

The widespread use of chemical compounds in daily life necessitates rigorous DNT testing. Current *in vitro* and animal tests often face challenges in translating results to

human applications due to ethical and practical limitations [119]. Chemical compounds are widely used in human life, while current DNT testing results for risk assessment from *in vitro* and animal tests are usually difficult to validate in humans because of a lack of adequate testing opportunities that circumvent practical and ethical constraints [119]. Consequently, advancements in hiPSCs-derived brain models have gained attention in scientific research and are used across various disciplines. Recent studies have experimentally demonstrated that hiPSCs can differentiate into various cell types, making them a valuable tool for identifying toxic substances. The effectiveness of hiPSCs in predicting neural toxicity has been confirmed by testing their response to known neural toxins like retinoic acid [14,37,95,98,99,120-128]. Additionally, several studies have shown the feasibility of using brain organoids for evaluating the potential neurotoxic effects of environmental chemicals [72,129-134]. The introduction of iPSC-derived microfluidic chip brain technology shows unprecedented advantages in neurotoxicity testing, which highly simulates the complex physiological environment of human brain cells [69,117,135-139]. These brain chips hold potential as a new chemical hazard assessment tool, allowing the development of a set of biomarkers that can be effectively detected to reliably screen hazardous chemicals and monitor brain toxicity induced by contaminants.

One of the major limitations of most current human *in vitro* models is the absence of vascular and glial components. It is crucial for DNT to incorporate microglia cells into *in vitro* models as they play an important role in the developing brain and are key for neuroinflammation, a crucial tissue response to environmental stress. In addition, astrocytes are critically involved in various central nervous system disorders, both as protectors against brain damage and as instigators of disease initiation and progression [140]. BBB is another essential component to include in neurotoxicity and DNT studies [60]. To date, advanced strategies to produce vascularized and glial *in vitro* models are actively being explored [69,127,132,133,135,141-147].

The prevailing view today is that a combination of epigenetic factors, environmental influences, and the interplay between genes and the environment contributes to the elevated risk of neurodevelopmental disorders such as autism. For example, Modafferi, et al. have recently addressed the complex interplay between genetic and environmental factors in ASD by using CRISPR/Cas9-engineered CHD8 heterozygous knockout and iPSCs brain organoids [109]. The special-temporal signalling may be possible by implementing microfluidic systems, growth factor gradients, and biomaterials such as ECM and BBB [148]. The combination of iPSC-*in vitro* models and omics data enables the building of a toxicity testing system for screening neural toxicants on a large scale before their widespread application to avoid the potential for toxicity and neurodevelopmental toxicity [113]. Furthermore, by exploring the potential of iPSC-derived models, we can establish a robust and high-throughput platform for screening environmental toxicants. By synergistically integrating functional evaluation, multi-omics data, and machine learning algorithms as well as morphological analysis, this novel platform could yield comprehensive and reliable screening capabilities for identifying environmental toxicants with unprecedented efficiency and accuracy. The incorporation of Next-Generation Sequencing (NGS) in iPSC disease modelling fulfils the need for genome sequencing with high cost-effectiveness and establishes a real-time correlation between transcriptomic profiling and phenotypic activities. It allows comparison between the sequencing outputs and the *in vitro* cytopathies, providing deeper insights into toxicological mechanisms [149]. HCS application approaches combining automated microscopy, quantitative image analysis, and iPSC-derived models are widely used for studying the mechanisms of compound toxicity, including developmental neurotoxicity and genotoxicity [150]. Li, et al. recently developed an HCS-based neurite outgrowth assay using iPSC-derived neurons expressing GFP. This innovation facilitates detailed live and time-lapse imaging of neurite outgrowth, enhancing the precision and depth of neurotoxicity assessments [95].

High-throughput single-cell RNA sequencing (scRNA-seq) characterizes the sequential changes of the transcriptome in individual cells. Therefore, in neurotoxicity research, scRNA seq can be employed to identify possible alterations in the developmental trajectories of neural cells at each time point of the differentiation process [151]. Multi-organoid-chip (MoC) platforms are equipped with combinations of multiple organoids, including the brain. This system can establish a complex process of chemical metabolism and responses through organs-organs interactions and, therefore, can be utilized to evaluate systemic toxicity from the dynamic process of distribution, absorption, metabolism, and excretion features of toxins in microfluidic systems [152].

Additionally, improving iPSC models is urgently needed to achieve improved reproducibility and standardization, reduced costs, increased throughput, and assay optimization. Further improvements are expected for more physiologically relevant models incorporating cellular components such as immune, glial, vascular, and genetically engineered cells. Furthermore, it would also be interesting to include iPSC-derived patient models to evaluate gene-environmental interactions (e.g., idiopathic autism) broadly. Last, the use of Artificial Intelligence (AI) emerges as a new promise to revolutionize toxicity. The DeepTox algorithm is an example that uses machine learning and the history of predefined toxicophore features to predict 12,000 environmental chemicals and drugs with high accuracy [153]. Furthermore, the advent of digital image analysis coupled with deep learning AI model provides an innovative approach to making quantification of neurotoxicity data automated and time-consuming [154].

These various models result from new technologies of exploration; although they are relevant, they do not replace the evaluations of the effects of early *in utero* exposures, which capture the critical processes of maternal-fetal chemical transfer, including the role of the placental barrier and maternal physiology [155]. In addition, *in vivo* observations are essential for tracking the emergence of sensory, behavioral, and cognitive functions at specific developmental stages, enabling the early detection of potential neurodevelopmental anomalies [156]. While *in vitro* models offer high-throughput and ethically favorable testing options that support the principles of the 3Rs (Refine, Reduce, Replace), real-world exposures are complex, often involving multiple agents that interact in ways difficult to isolate in a controlled environment. Therefore, an integrated approach—leveraging both *in vitro* technologies for molecular insights and *in vivo* models for comprehensive, systemic effects—holds the greatest promise for accurate neurotoxicity assessment [81]. Such a combined strategy enhances the predictive value of neurotoxicity evaluations, providing a precise and realistic understanding of how cumulative environmental exposures affect human neural development.

Conclusion

iPSC-derived brain models have profoundly revolutionized neurotoxicology, advancing the fields of basic research, chemical risk assessment, and translational medicine. By closely replicating human neural physiology, these models serve as a crucial bridge between fundamental research and clinical application, enabling more accurate evaluations of neurotoxicity risks associated with environmental substances and pharmaceutical compounds. Their relevance extends beyond general toxicology, providing a pathway to personalized risk assessment by evaluating individual susceptibility to neurotoxic agents. This capability facilitates precision interventions tailored to specific genetic and environmental profiles, thus advancing the field of personalized medicine.

Moreover, iPSC-derived models enable the dissection of neurotoxic mechanisms at the molecular level, underlying neurotoxic effects, which is essential for the development of targeted therapeutic interventions. Their application in drug development is equally transformative, with the potential to improve early-stage neurotoxicity detection and reduce adverse effects, thus enhancing the safety profile of candidate drugs prior to clinical trials. The advent of high-throughput, automated screening platforms based on iPSC-derived models further strengthens their utility, providing robust, scalable, and ethically sound options for screening a wide array of environmental and food-related toxicants.

The integration of these human-relevant models with *in vivo* approaches and advanced in silico tools provides a comprehensive framework for neurotoxicity research and risk assessment. Leveraging the predictive capabilities of these models in regulatory toxicology will be crucial for advancing human health protection and supporting personalized therapeutic strategies. This paradigm shift not only promises to safeguard public health from environmental hazards but also accelerates the transition toward individualized therapeutic treatments. By refining risk-assessment methodologies, iPSC-derived brain models stand at the forefront of innovation in toxicology and personalized medicine, underscoring their potential to revolutionize both fields and drive meaningful advances in human health protection and therapeutic development.

Author's contribution

Narimane Kebieche: Conceptualization, Methodology, Writing. Farzana Liakath Ali and SeungAe Yim: Reviewing and Editing. Mohamed Ali: Reviewing. Rachid Soulimani. Writing and Supervision. Claude Lambert: Supervision.

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