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Review article

Impact of environmental neurotoxic: current methods and usefulness of human stem cells

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ABSTRACT

The development of central nervous system is a highly coordinated and complex process. Any alteration of this process can lead to disturbances in the structure and function of the brain, which can cause deficits in neurological development, resulting in neurodevelopmental disorders, including, for example, autism or attention-deficit hyperactivity disorder.

Exposure to certain chemicals during the fetal period and childhood is known to cause developmental neurotoxicity and has serious consequences that persist into adult life. For regulatory purposes, determination of the potential for developmental neurotoxicity is performed according the OECD Guideline 426, in which the test substance is administered to animals during gestation and lactation. However, these animal models are expensive, long-time consuming and may not reflect the physiology in humans; that makes it an unsustainable model to test the large amount of existing chemical products, hence alternative models to the use of animals are needed.

One of the most promising methods is based on the use of stem cell technology. Stem cells are undifferentiated cells with the ability to self-renew and differentiate into more specialized cell types. Because of these properties, these cells have gained increased attention as possible therapeutic agents or as disease models.

Here, we provide an overview of the current models both animal and cellular, available to study developmental neurotoxicity and review in more detail the usefulness of human stem cells, their properties and how they are becoming an alternative to evaluate and study the mechanisms of action of different environmental toxicants.

1. Introduction

Developmental disorders affect millions of people and have a significant impact on their life, their families and the societies to which they belong. The prevalence of disorders such as Autism Spectrum Disorders, Attention Deficit Hyperactivity Disorder and subclinical decrements in brain function, are increasing worldwide (Yap et al., 2015; Grandjean and Landrigan, 2006). Although these disorders are associated with alterations in some genes, it is known that genetics exclusively is not the cause, and the exact etiology remains unknown (Landrigan et al., 2012). The general population is continuously exposed to potential neurotoxic compounds in daily life, from chemicals in products for human consumption to environmental pollutants (i.e. cosmetic, cleaning products, medicines, pesticides, air pollution particulate matter, flame retardants, mercury, lead, etc). This exposure, especially during prenatal and early postnatal life, is one likely explanation for the appearance of certain behavioral disorders and decreased brain function later in life (Maffini and Neltner, 2015).

Existing scientific evidence shows that the immature nervous system may be more susceptible to chemical exposure compared to the adult nervous system (Bal-Price et al., 2018a). The formation of the nervous system is unique when compared to other systems. It begins with embryogenesis, continues throughout the fetal period and, unlike most other systems, continuing after birth. For these reasons, the brain is especially vulnerable to toxic exposure occurring at any time during its developmental process (Heyer and Meredith, 2017).

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These complex and specific processes involved in brain development include the commitment of neural progenitor cells followed by proliferation, migration, and differentiation into several glial and neuronal subtypes, synaptogenesis, myelination, neuronal network formation and terminal glial and neuronal maturation (Stiles and Jernigan, 2010; Rice and Barone, 2000; Fritsche et al., 2018a). In the assessment of chemical substances causing developmental neurotoxicity (DNT) it is important not only the type of exposure (dose, duration, etc), but also on the stage of development at the moment of exposure. In addition, the blood-brain barrier (BBB) is not fully formed after birth, which facilitates the entry of some chemicals into the fetal/neonatal brain (Landrigan and Goldman, 2011). Disruption at any point during the development process may lead to severe and long-lasting disturbances on the children and adult brain (Heyer and Meredith, 2017); thus, resulting in neurodevelopmental disorders (NDD), with the most likely effects being learning disabilities, cognitive impairment, developmental delays and cerebral palsy.

Furthermore, children have greater energy demands than adults, which means that they breathe faster and have a higher relative intake than adults (Koger et al., 2005; Weiss et al., 2004). With regard to diet, infants can be ingesting toxic lipophilic substances accumulating in maternal adipose tissue through breast milk (Grandjean and Landrigan, 2006; Weiss et al., 2004). Children often spend more time playing outdoors and they touch and suck the objects that are on the ground (Ferguson et al., 2017; USEPA, 2013). All these reasons make them more vulnerable to environmental toxics. In fact, it has been proved that, under the same environmental exposure, toxics reach highest levels in the blood of children than in adults (Miodovnik et al., 2011).

The increasing amount of pollutants in our environment, as a product of industrial and agricultural procedures (Le Magueresse-Battistoni et al., 2018), and the mixtures of such compounds, creates a growing concern for the effect they can have on public health, particularly at vulnerable stages of development (Kienzler et al., 2016; Carvalho et al., 2014). This is aggravated because the chemical risk assessment approach is traditionally based on the toxicity caused by a single chemical on a variety of organs; however, we are currently exposed to multiple chemicals that may have cumulative biological effects on the same organ or system (Kienzler et al., 2016; Maffini and Neltner, 2015). However, the major challenge in this field is the lack of accurate DNT data for existing chemical compounds (Fritsche et al., 2018b). At a regulatory level, systematic testing of DNT is not a standard requirement within the EU or USA chemical legislation safety assessment (Bal-Price et al., 2018b). In certain cases, specific DNT tests are performed after observing neurotoxic effects in *in vivo* animal tests with repeated doses, a known neurotoxic mode of action or a structural activity alert (Bal-Price et al., 2018b). In these cases, DNT assays are with animal models (mainly according to OECD guideline 426). These studies are costly, time consuming and the results are not always reliable to assess the impact of chemical compounds on the developing human brain (Aschner et al., 2017; Terron and Bennekou, 2018; Beronius et al., 2013 Paparella et al., 2020), due to the fact that animal models do not perfectly reflect human physiology (Bal-Price et al., 2015, 2018a). It remains clear that there is a growing necessity for developing alternative methodologies that can better identify and assess chemical substances with the potential to induce neurotoxicity during brain development and maturation (Bal-Price et al., 2011; Fritsche et al., 2017). International efforts to enhance DNT testing have been recently revised (Sachana et al., 2019) and currently, there are no alternative methods accepted for this purpose at the regulatory level.

In order to use the alternative methods for DNT in a regulatory context it is necessary to define readiness criteria. A very comprehensive review that proposes a first approach to this matter was published in 2018, with consensus from scientists, industry, and regulatory authorities Bal-Price et al. (2018b). The criteria were clustered into 13 groups concerning to test system, exposure scheme, documentation, main endpoint, cytotoxicity, test method controls, data evaluation, testing strategy, robustness, test benchmarks, prediction model, applicability domains and screening hits. The authors proposed a scoring system to obtain indications on the readiness status of various published DNT test methods.

It's also important to examine which chemical substances known to be associated with a DNT effect have been correctly o incorrectly identified by the alternative methods. In a very useful and interesting review (Aschner et al., 2017), criteria for the selection of positive and negative controls have been defined. Also, a broad set of compounds have been compiled, as well as guidelines on how to use them to address the specificity, predictability and utility of alternative methods for different endpoints related to DNT. In Table 1 we included some examples of studies that have tested some of these chemical substances in the alternative methods listed in this review.

In vitro systems are becoming a powerful tool to evaluate the toxicological effects of chemical compounds on DNT with promising results. Several cell lines, including tumor and immortalized cells, are currently being used as models for toxicity screening assays. These cell lines are easily accessible and maintained, but they are not capable of reproducing human brain development. Stem cells are currently being a model that promises to be very useful in evaluating this type of toxicity. Stem cells are undifferentiated cells with the potential to differentiate into more specialized cell types. They are present during brain development, which therefore makes them a more appropriate model to mimic key events that take place during embryonic development.

In this review, we summarize and discuss current models, both animal and cellular, used to assess study DNT. Finally, we give an overview of the different types of stem cells, their properties and how these cells are becoming an important alternative to DNT studies, including, three-dimensional (3D) cell cultures.

2. Current models for DNT assessment

2.1. Animal models

2.1.1. Mammalian species: rat/mice

The current regulatory standard DNT testing are based on animal assays according to OECD TG 426 (OECD TG 426, 2007) and similar standardized protocols. This is a specialized type of developmental toxicity study designed to screen for adverse effects of pre- and postnatal exposure on the development and function of the nervous system. It also provides dose response characterization of those outcomes. The test substance is administered to pregnant female rats from gestation day 6 to postnatal day 21. The study requires 20 animals per sex and group. Functional tests (including motor activity, learning and memory, functional observation battery and startle response), brain weight, and neuropathology tests, are carried out from postnatal day 4 to postnatal day 60.

Only approximately 100–150 chemicals have been assessed using regulatory DNT guidelines, with the majority being pesticides (Sachana et al., 2019). Compared with the thousands of chemicals that humans may be exposed to, this number of assays is too low.

After conducting and evaluating DNT studies over the last two decades, several problems have been identified (Tsuji and Crofton, 2012), a summary of limitations and uncertainties has recently been published (Paparella et al., 2020). Studies performed are low-throughput and different techniques employed do not provide comparable results because they do not show a reliable model to assess the impact of chemical compounds on the developing human brain (Bal-Price et al., 2018a). Learning and memory tests are complicated and require more technical support or involve longer periods of time for training and testing. Morphometric examination of nervous system tissues requires fixation and embedding in paraffin or plastic block, and great care must be taken to ensure concordance among sections from different animals (i.e. maintaining proper orientation in all three dimensions when slicing tissues).

As with all data from laboratory animal studies, extrapolation of results to humans is fraught with uncertainties. Some of these are specific

Table 1. Advantages and inconveniences of *in vivo* and *in vitro* models in developmental neurotoxicity (DNT) assays.

DNT ASSAYS			
In vivo models			
	ADVANTAGES	INCONVENIENCES	REFERENCES*
Standard rodent *in vivo* DNT studies	Regulatory validated test Complexity of complete organism (ADME processes) Relevant route of administration Defined critical endpoint to derive limit values for human health	High cost Ethical aspects No mechanism information Anatomy development differences Species differences for molecular signaling and cell differentiation Complexity in study design High expertise	Makris et al. (1998); Makris et al. (2009); Phang, 2003; Raffaele et al. (2010);
Non-mammalian	No ethical problems Identification of mechanisms of action Technical advantages ADME processes ↓ Cost	Species differences No harmonized protocol	
Danio rerio (zebrafish), early developmental stages	Genome homology with humans (≈70%) Signal-transduction mechanisms, anatomy and physiology homologous to humans Many brain subdivisions identifiable Neurotransmitters; GABA, glutamate, serotonin, dopamine, noradrenalin, acetylcholine Useful behavioral assays	Route of exposure no relevant (chemical added to aqueous media) Subtle differences in metabolic activity Primary sequences not necessarily highly conserved	Chen et al. (2012a); He et al. (2016); Selderslaghs et al. (2013); Jin et al. (2016); Eddins et al. (2010); Fan et al. (2010)
Drosophila melanogaster	Short life span Large number of offspring Many genetic techniques available Well-known anatomical Wide variety of mutants CNS composed of neuros and glial cells Fundamental molecular pathways are highly conserved	Route of exposure no relevant Subtle differences in metabolic activity Primary sequences not necessarily highly conserved Anatomic divergence	Bonilla-Ramirez et al. (2011); Seong et al. (2017); Algarve et al. (2020)
Caenorhabditis elegans	High reproductive rate Small size Capacity of growing synchronization (through temperature control), allow hundreds of worms in the same developmental stage Self-fertilizing hermaphrodite (generation of homozygous mutations) Genome homology with humans (≈80%) Biochemical pathways highly conserved between *C. Elegans* and mammals Similar neurotransmitters Exhibits social interaction Transparent during development	Impossibility of developing sex-related approaches Evolutionary differences with humans Anatomic divergence	Roh et al. (2016); Helmcke et al. (2010)
***In vitro* models**			
Cell cultures	↓ Cost ↑Throughput ↑Replicates ↑Concentration range ↑Comparability of results Mechanistic information	No harmonized protocol Limited metabolism Necessary models to extrapolate vitro to *in vivo* results No defined critical endpoints to derive limit values	
Primary cells	Maintained in cultures several passes Well-defined Isolated from brain's animals: closely replicate Reproduce events of developing brain: proliferation, migration and differentiation	Heterogeneous: low reproducibility Difficult to maintain in culture for a long time Isolated from brain's animals: ethical problem	Hogberg et al. (2009); Harry et al. (1998); Radad et al. (2006); Bollimpelli and Kondapi (2015); Slotkin et al. (2016)
Murine tumor cell lines	Well-defined	Accumulation of genetic alterations after long-term passage Extrapolated murine cells to human cells	PC12 cells: Greene and Tischler (1982); Parran et al. (2003); Roth et al. (2002); Crumpton et al. (2001); Christen et al. (2017); Shou et al., 2019; Xu et al., 2012; Lee et al. 2014; Hu et al. (2015)
Human tumor cell lines	Well-defined Physiology of human ells	Accumulation of genetic alterations after long-term passage	SH-SY5Y cells: Attoff et al. (2016); Hong et al. (2003); Gandhi et al. (2016); Tarale et al. (2017) NTERA2.cl.D1 (NT2): Menzner and Gilbert (2017); Stern et al. (2014); Laurenza et al. (2013)

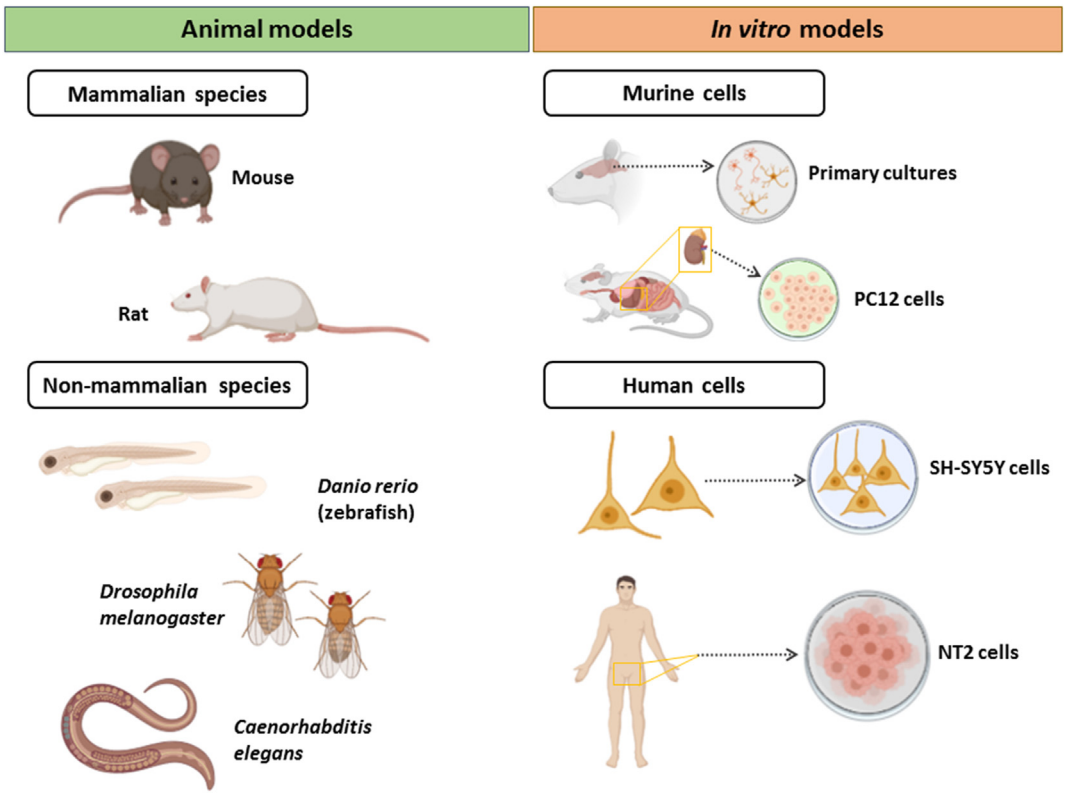

Figure 1. Schematic representation of the different models mentioned in the review for developmental neurotoxicity assessment.

to DNT studies, probably due to differences in physiology and metabolism (Rice and Barone, 2000; Clancy et al., 2007). A major uncertainty is the timing of growth of the different regions of the brain (Dobbing and Sands 1979; Howdeshell 2002). Due to these differences, the most sensitive critical windows of exposures for similar brain regions can be very different between animals and humans. The major brain growth spurt is around the postnatal day 7 in rats, while it is around birth in humans (Dobbing and Sands, 1979; Howdeshell, 2002).

Thus, DNT guideline studies are expensive, time consuming, require large numbers of animals and does not provide mechanistic information. In addition, there is a limited set of laboratories that can conduct these types of studies. For these reasons, the current *in vivo* DNT testing are not sufficient to evaluate chemicals that might have effects on the developing human brain.

2.1.2. Non-mammal species

Attempts have been made to use other whole organisms, not mammals, so that the complexity of complete organisms is addressed taking into account processes such as absorption, distribution, metabolism and excretion (ADME), as well as homeostasis mechanisms that do not reproduce in a cell culture. *Caenorhabditis elegans* (a nematode), *Drosophila melanogaster* (a fruit fly), and *Danio rerio* (zebrafish), have proven being useful for the detection of neurotoxic substances and the identification of mechanisms of action. Many technical advantages are shared among the three non-mammal species described here (Figure 1).

2.1.2.1. Danio rerio (zebrafish). Although adult zebrafish are being widely used, especially for translational research (Phillips and Westerfield, 2014; Pickart and Klee, 2014), early stages of zebrafish life are particularly suitable for neurodevelopmental studies, furthermore at early developmental stages are considered non-animal testing (Directive, 2010/63/EU, 2010).

In zebrafish during the first 5 days post fertilization (dpf), almost all gene products and signal transduction pathways are expressed (Pauli et al., 2012); at this stage of early life it also expresses a complete battery of phase I and phase II metabolism systems, whose activities are similar to those of humans (Goldstone et al., 2010). Many brain subdivisions found in the developing mammalian brain are identifiable in the developing zebrafish, and neurotransmitters including GABA, glutamate, serotonin, dopamine, noradrenalin, and acetylcholine are found in the neurons of zebrafish at 1–5 dpf (Panula et al., 2010).

The stereotypic motor activity of the developing zebrafish includes three sequentially appearing behaviors that are in line with neurodevelopment: a transient period of alternating tail coiling followed by responses to touch, and the appearance of organized free swimming of larvae (Nishimura et al., 2015). These behaviors appear comparable at a functional level with human behavior, with links to neural circuitry underlying. Behavioral assays for DNT in zebrafish have been widely used (Chen et al., 2012a; He et al., 2016; Selderslaghs et al., 2013; Jin et al., 2016). However, there is a need for a harmonized protocol (Legradi et al., 2015).

2.1.2.2. Drosophila melanogaster. Modelling human brain diseases in *Drosophila melanogaster* offers several advantages for investigation of molecular and cellular mechanisms underlying human diseases. Short life span, large number of offspring, many genetic techniques, a well-known anatomical situation and a wide variety of mutants are convenient characteristics of *Drosophila* as a model organism. Furthermore, the central nervous system (CNS) of the *Drosophila* embryo is composed of neurons and glial cells (Jeibmann and Paulus, 2009). Although, anatomic divergence between *Drosophila* and humans is apparent, fundamental molecular pathways are highly conserved (Rubin and Lewis, 2000).

Several studies have employed *Drosophila* as a model to assess neurotoxicity effects of different environmental pollutants, including metal ions (iron, manganese and copper) (Bonilla-Ramirez et al., 2011), dichlorodiphenyltrichloroethane (DDT) (Seong et al., 2017), or methylmercury (Algarve et al., 2020).

2.1.2.3. Caenorhabditis elegans (C. elegans). *C. elegans* is a nematode widely used in the research of human diseases. This invertebrate model has several advantages (Ruszkiewicz et al., 2018; Queirós et al., 2019) (see Table 1). Approximately 80% of their genes have homologous in humans and many biochemical pathways are highly conserved between *C. elegans* and mammals, which makes findings greatly extrapolated (Ruszkiewicz et al., 2018).

C. elegans has similar neurotransmitters and associated receptors to mammals, and toxics usually provoke movement changes in these worms. Apart from the existing conservation of the neural pathways, *C. elegans* exhibits social interaction and is transparent during development (Silva, 2020).

An example in which *C. elegans* is exposed to an environmental toxic for analyzing its neurotoxicity is the study developed (Roh et al., 2016) with the pesticide chlorpyrifos (CPF).

2.2. In vitro models

During the last decade, several *in vitro* models have become important as good alternatives for studying how chemical substances affect brain development. These include, primary cultures derived from animal models, tumor cell lines, immortalized cells and human stem cells.

2.2.1. Murine cells

In this type of *in vitro* models, we can highlight primary cultures and cell lines. Primary cultures are prepared directly from brains isolated from animals and they are one of the most widespread *in vitro* models, which reproduce several events that take place in the developing brain, such as proliferation, migration and differentiation. For example, primary cultures from rat cerebellar granule cells (CGCs) have been broadly used in the field of DNT to closely replicate crucial stages of neurodevelopment (Hogberg et al., 2009; Harry et al., 1998). Another example is primary culture of ventral mesencephalic dopaminergic neurons that have been used to study neuronal toxicity of several pesticides in increasing the risk of developing neurodegenerative disorders such as Parkinson's disease (Radad et al., 2006; Bollimpelli and Kondapi, 2015).

Primary neural progenitor cells isolated from rat cortical neuroepithelium on embryonic day 14 have been used to analyze the effects on the ability to differentiate into neurons or glia cells of several chemicals such as the glucocorticoid (dexamethasone), organophosphate insecticides (CPF, diazinon,parathion), insecticides targeting the $GABA_A$ receptor (dieldrin, fipronil), heavy metals (Ni^{2+} and Ag) and nicotine (Slotkin et al., 2016).

Murine tumor cell lines such as the PC12 cells (a cell line derived from pheochromocytoma of the rat adrenal medula) (Greene and Tischler, 1982) have been widely employed to study neural growth and neurotoxicity of different pesticides (Parran et al., 2003; Roth et al., 2002; Crumpton et al., 2001; Christen et al., 2017) like for example CPF and tetrachlorobenzoquinone, a reactive metabolite of pentachlorophenol (Hu et al., 2015).

2.2.2. Human tumor cell lines

Since the physiology of human cells is different from that of murine cells, currently, *in vitro* models of human cells are considered the best option to study DNT. These include tumor cell lines and stem cells (*see next section*).

Several well-defined tumor cell lines have been used for neurotoxicological studies. One of the most used lines are the **SH-SY5Y cells** (a human neuroblastoma cell line) that have been employed to study neurotoxic effects of different compounds like acrylamide (Attoff et al., 2016), several organophosphorus (Hong et al., 2003) and the genomic and proteomic effects of **e**ndosulfan, an organochlorine pesticide (Gandhi et al., 2016), as well as DNA methylation alteration by manganese (Tarale et al., 2017).

Another cell line commonly used as a model of neural cell development is **NTERA2.cl.D1 (NT2),** a cell line derived from a human testicular embryonal carcinoma (Przyborski et al., 2004). Human NT2 cells are considered a suitable model for *in vitro* developmental toxicity and neurotoxicity studies as they undergo neuronal differentiation upon stimulation with retinoic acid and allow toxicity assessment at different stages of development (Menzner and Gilbert, 2017). NT2 cells showed sensitivity to the well-known neurotoxicants methylmercury chloride (MeHgCl), sodium arsenite, sodium valproate and methylazoxymethanol in a DNT screening test, neuronal differentiation and migration of NT2 cells being reduced (Stern et al., 2014). In a long-term exposure experiment, NT2 cells differentiated to glial or neuronal phenotype were treated with lead chloride, aluminum nitrate and MeHgCl. Results denoted differential sensitivity to neurotoxicants between mature neural phenotypes, posing glial and neuronal mixed cultures derived from NT2 cells as an adequate model for DNT assessment (Laurenza et al., 2013). Also, NT2 cell line was used to evaluate whether microRNAs (miRNAs) could be a valuable tool for DNT testing. Since miRNAs are involved in crucial developmental processes such as neurogenesis, neurite outgrowth and neuronal differentiation, the changes in miRNAs could serve as a promising DTN biomarker (Pallocca et al., 2013).

Nowadays, the best alternative to the employment of these tumor lines would be the use of human stem cells, in particular, human pluripotent stem cells (hPSCs). Special interest has been focused on *in vitro* neuronal cultures obtained from these stem cells since they have the ability to differentiate towards neuronal and glial phenotypes and can recapitulate human brain development (Bal-Price et al., 2018a). Stem cells have emerged as a promising alternative for *in vitro* studies because they provide an unlimited source of cells and can be kept in culture for long periods of time, and therefore can serve as chronic models of toxicity.

3. Human stem cells (types and properties)

Stem cells are undifferentiated cells with the ability to self-renew and differentiate into more specialized cell types. They can be classified attending to their differentiation potential into: totipotent, pluripotent, multipotent and unipotent as demonstrated by their capacity to generate a variety of cell lineages (Singh et al., 2016). However, the most used nowadays are: pluripotent and multipotent stem cells. Pluripotent stem cells are non-committed cells able to give rise to specialized cell types from all three germ layers (Bongso et al., 2008; Martinez-Morales and Liste, 2012), while multipotent stem cells are committed to form different cells of the same tissue (Martinez-Morales et al., 2013) (Figure 2).

Because of their properties, these cells have earned increased attention as potential therapeutic agents and could be used to treat many chronic diseases, such as Parkinson's disease, diabetes or injury of the spinal cord. Furthermore, the application of stem cell research may well provide a useful tool for a better understanding of drug-induced adverse reactions and toxicological tests (Brunt et al., 2012).

3.1. Human multipotent stem cells: human neural/progenitor stem cells

Although qualitative differences do exist between neural progenitors (NPs) and neural stem cells (NSCs), these are difficult to distinguish and thus one or another terminology is often used indiscriminately (Liu and Wang, 2018). Human Neural Stem Cells (hNSCs) are multipotent stem cells defined by their ability to self-renew and differentiate into all cells of the CNS (i.e. neurons, astrocytes and oligodendrocytes). These cells can be obtained from the fetal, neonatal and adult brain (Martinez-Morales et al., 2013). Further, hNSCs can be obtained from two major types of pluripotent stem cells (PSCs), human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). Taking into consideration the ethical issues and the differences in national legislation regulating the generation and use of hESCs and/or fetal-derived tissues, hiPSC-derived neuronal and glial models are currently gaining increasing scientific interest for their applicability in a broad range of *in vitro*

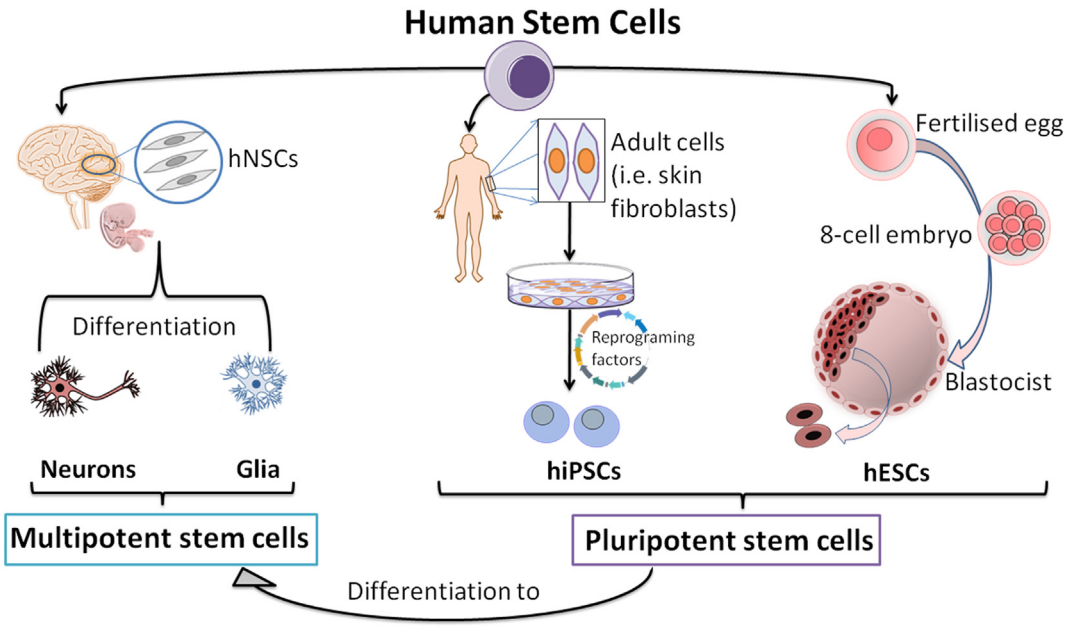

Figure 2. Schematic representation of the types of Human Stem Cells according to their differentiation potential.

pharmacological and toxicological studies, including DNT (Bal-Price et al., 2018a).

hNSCs can be propagated *in vitro* as floating undifferentiated aggregates, called neurospheres, containing a mixture of self-renewing stem cells and more restricted progenitor cells, in the presence of growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF2). Neurospheres have been utilized to evaluate DNT since they can reproduce key processes of human neurodevelopment, including proliferation, *in vitro* differentiation and migration (Kim et al., 2009; Moors et al., 2009). The major limitation of this model is the absence of a BBB and certain enzymes that form natural physiological barriers against toxic exposure (Grandjean and Landrigan, 2014). Despite this limitation, hNSCs are a promising model for DNT screening assays (Bose et al., 2012). The unique properties of progenitor cells make them a useful tool to evaluate neurodevelopmental effects of environmental pollutants such as pesticides (Lee et al., 2014; Tiethof et al., 2018), metals (Oh et al., 2016; Wagner et al., 2016; Jiang et al., 2017), and even water disinfection byproducts (Fu et al., 2017).

However, a limitation associated with the use of human neurospheres is that they can only be expanded by a limited time, and they also lose their initial properties with passages in culture.

These problems can be avoided at least in part, by the immortalization of hNSCs which can provide relatively homogeneous cultures and give the opportunity to generate genetically-modified subclones of interest. One of the most available immortalized cell lines are Lund human mesencephalic **(LUHMES)** cells. LUHMES are a subclone of human mesencephalic derived cell line MESC2.10, conditionally immortalized using a tetracycline-regulated v-myc gene (Lotharius et al., 2005). LUHMES are considered as a strongly reliable neuronal model used in neurodevelopmental studies but also appear to be an adequate model for assessing neuronal cytotoxicity. The organophosphate pesticide CPF increased mitochondrial dysfunction and apoptotic cell death in LUHMES cells at low exposure concentrations, raising these cells as a good model for neurotoxicity assessment especially because of their great sensitivity to cytotoxicity (Singh et al., 2018).

Other example of human cell line used is ReNcell CX, an immortalized human neural progenitor cell line derived from 14-week gestation human fetal cortex (Donato et al., 2007). These cells have been successfully employed to analyze the effects on proliferation and viability of different developmental neurotoxicants (Breier et al., 2008).

Another immortalized cell line of hNSCs are the hNS1 cells. This is a clonal line derived from the telencephalic region of the developing brain and immortalized with the oncogene v-myc (Villa et al., 2000). These cells present self-renewal capacity in the presence of mitogens (FGF2 and EGF) and stop dividing upon mitogen removal, undergoing spontaneous morphological differentiation, expressing neuronal and astrocyte markers. There are several benefits of using these cells. Firstly, they present a homogenous cell population of human origin. They are clonal cells that maintain a stable phenotype over time, they are easy to grow and expand in culture and they differentiate easily to neurons and glial cells. Furthermore, they provide an unlimited supply of neural precursors, neurons and glial cells without the need for primary tissue preparation. Finally, they are a good source of cells to be used in standardized, *in vitro* studies for drug discovery and research applications (Villa et al., 2000, 2004).

In previous results in our laboratory we have used hNS1 cells to test the effects of the pesticide CPF on cell death, cell proliferation and cell fate specification of these cells under differentiation conditions. The results showed that this compound induces apoptotic cell death at the highest doses tested. Besides, CPF promoted the differentiation of hNS1 cells into glial cells by increasing the pool of proliferating glia progenitors without affecting neurogenesis. This effect may be associated with a protective effect of glia against CPF (Sandoval et al., 2019).

3.2. Human pluripotent stem cells

hPSCs include both **hESCs and hiPSCs.** Along with the development of new technologies, hPSCs are emerging as a powerful tool for DNT screening.

hESCs were first isolated from the inner cells mass of the blastocyst in 1998 by James Thomson (Thomson et al., 1998). These cells are characterized by their potential to self-renew over long periods of time and they can give rise to differentiated cell types of the primary germ layers (ectoderm, mesoderm and endoderm) (Henningson et al., 2003). Since these cells have the potential to differentiate into all body cell types, hESCs may constitute an almost limitless ideal cell source for cell-based therapies. Cryopreservation and differentiation protocols for analyzing the effect of numerous neurotoxicants in a wide range of hESC-derived cell types are well stablished (Schmidt et al., 2017). Using hESCs differentiated towards midbrain dopaminergic neurons or cortical

neurons showed the toxicological effect of the commonly used pesticide propargite (Zhou et al., 2018). This research indicates that propargite is able to induce cell death in human neurons, similar to the results obtained for pancreatic β-cells and also points out that midbrain dopaminergic neurons are much more sensitive to propargite than cortical neurons. These data could explain the association observed between the levels of contamination with propargite and the increase in the prevalence of Parkinson's disease in some places (Zhou et al., 2018). This study provides a good example of a hESC-based *in vitro* system adequate for toxicological assessment of environmental factors.

In another study, using a hESC differentiation model, Chen et al. investigated if polybrominated diphenyl ethers (PBDE), a sort of halogenated compound that has emerged as an important environmental pollutant, could cause neurodevelopmental toxicity at different stages of neuronal development. They found that PBDE induces cytotoxicity and inhibits several processes like growth and proliferation. Besides, the susceptibility of this differentiation model to PBDE depends on the stage of development in which exposure occurs (Chen et al., 2019).

Unfortunately, ethical controversy strongly limits research using hESCs because they are obtained from human embryos (De Miguel-Beriain, 2015).

In a recently research, the authors develop an improved variant of the human stem cell-based test STOP-tox$_{(UKN)}$ to investigate if the rosette formation could be employed as an endpoint for studying DNT. They found that rosette formation is disturbed when toxic substances are present (Dreser et al., 2020).

In the last decade, several reprogramming techniques have been developed to reprogram adult somatic cells to a state of pluripotency, generating the so-called hiPSCs (Takahashi et al., 2007). This novel discovery now allows scientist to circumvent the ethical controversies involved in the isolation and use of hESCs (Brouwer et al., 2016). iPSCs were first obtained by Takahashi and Yamanaka in 2006 from mouse fibroblast through the overexpression of four reprogramming transcription factors: Oct 3/4, Sox2, Klf4 and c-Myc (known as "OSKM factors") (Takahashi and Yamanaka, 2006). A year later, in 2007, Yamanaka's group used the same reprogramming method for adult human fibroblast to generate hiPSCs (Takahashi et al., 2007) and James Thomson's team reported the generation of the same hiPSCs using two different factors, NANOG and LIN28 (Yu et al., 2007). hiPSCs have a similar expression pattern and morphology to hESCs from the source organism. In this regard, Hofrichter et al. demonstrated that MeHgCl, an organometallic toxicant, has an equivalent strong effect in the migration of both hiPSC-derivated NPs and primary NPs (Hofrichter et al., 2017). In a recently published investigation, researchers utilized hiPSC-derived NPs differentiated into mixed cultures of neurons and astrocytes to determine the effects of different treatments with single chemicals and mixtures belonging to diverse classes, all of them related to cognitive deficits in children. Their findings indicated that the use of hiPSC-derived mixed neuronal/glial cultures is a useful tool to identify chemicals with potential to cause NDD (Pistollato et al., 2020).

The use of hiPSCs overcomes the main problem related to the use of hESCs since they can be easily obtained from patients or healthy individuals in a non-invasive way. It is important to take into account that, when somatic cells are obtained from an adult individual, they will maintain the genetic background of the adult source after reprogramming. This is usually regarded as a disadvantage because genetic differences may influence the toxicological response of the cells. However, under directed conditions, this same diversity could be exploited to model specific genetic backgrounds in order to analyze their interaction with environmental factors and their combined potential to develop diseases (Luz and Tokar, 2018).

There is an increasing body of evidence that shows that most of the human diseases, including neurological disorders, are multifactorial and appear to be caused to an interaction between genetics and the environment. Thus, hiPSCs-based methods represent an ideal platform for rapidly assessing impact of environmental exposures on human CNS tissue.

hiPSCs could be derived from patients with neurodevelopmental disorders such as for example Rett syndrome or Down syndrome (Hogberg et al., 2013). Once the hiPSCs are generated and characterized, these cells, together with control cells can be differentiated into the desired neuronal or glial phenotypes. These cultures can be used to examine dysfunctional developmental processes as predicted by ported mutations and to analyze whether these genetic mutations increase sensitivity to different chemical compounds tested (Hogberg et al., 2013).

hiPSC-derived neurons and glia, even after long term culture, are unable to complete their maturation until adulthood. Therefore, these cultures constitute a prominent model for studying DNT (Bal-Price et al., 2018a). Considerable progress has been made in recent years in the differentiation protocols of human PSCs to obtain different neuronal and glial types. However, these cultures still need to be improved and optimized to achieve greater maturation in order to be capable of generating active and functional neuronal networks, similar to those observed *in vivo*.

As it has been reported for hESCs, hiPSCs can be easily cryopreserved and their differentiation protocols to diverse cell types (such as midbrain dopaminergic neurons, cortical glutamatergic neurons, cholinergic neurons, astrocytes, oligodendrocytes, etc.) are already stablished (Schmidt et al., 2017).

In a recently published study (Augustyniak et al., 2017) scientists tested the effect of pyrroloquinoline quinone (PQQ), a mitochondrial biogenesis stimulating agent, on hiPSCs-derived hNSCs at different neural stages of development. The authors differentiated hiPSCs upon distinct commitment stages (NSCs, early NPs [eNPs] and NPs) and showed that eNPs stage was the only one in which PQQ increased mitochondrial biogenesis, thus pointing out a specific "developmental window" of sensitivity to a certain chemical agent. Interestingly, distinct phenotypes were found between NSCs and NPs whereas eNPs showed intermediate characteristics between the other two groups, this providing scarce data to consider eNPs as a different population. However, differences found regarding mitochondrial biogenesis suggest that eNPs obtained by the protocols used in this work are in fact going through a metabolic change which is not necessarily paired with phenotypical changes but does represent a specific developmental stage. Although protocol standardization must be done, such a study brings a new approach to accurately mimic crucial stages of neural development which may be critical points for DNT.

Another work with hiPSCs was carried out to test if 5-fluorouracil (5-FU), an anti-cancer drug, has toxic effects on development. It was found that 5-FU reduce intracellular levels of ATP and the levels of mitofusin 1 and 2 (Mfn1/2), proteins that participate in mitochondrial fusion. These results suggest that 5-FU has neurotoxic effects on hiPSCs via Mfn and can be used as a potential marker of the toxicity of some drugs (Yamada et al., 2018).

hiPSCs seem to be a promising tool for toxicological studies since their potential to differentiate towards nearly any cell type of interest opens a wide range of possibilities. For example, Hoelting et al. differentiated hiPSCs to immature dorsal root ganglia neurons and exposed them to a wide range of compounds, in order to evaluate alterations on neurite growth. By adapting the available protocols to the necessities of their cells, this work provided a valuable model for drug and chemical assessment to identify peripheral neurotoxicant (Hoelting et al., 2016).

The discovery of both hESCs and hiPSCs provides new chances for neurotoxicology assessment, as well as for studying neural development and neurological disease mechanisms *in vitro* both in two-dimensional (2D) and 3D model systems (Figure 3).

Recently, it was established a hiPSC-based 3D system to test the effects of diverse toxicants with neurotoxic effects. Changes in differentiation, neurite outgrowth and apoptosis were evaluated. Results showed that hiPSC-based 3D *in vitro* neurosphere systems could be a valuable model to use for DNT studies and replace the use of animal models (Kobolak et al., 2020).

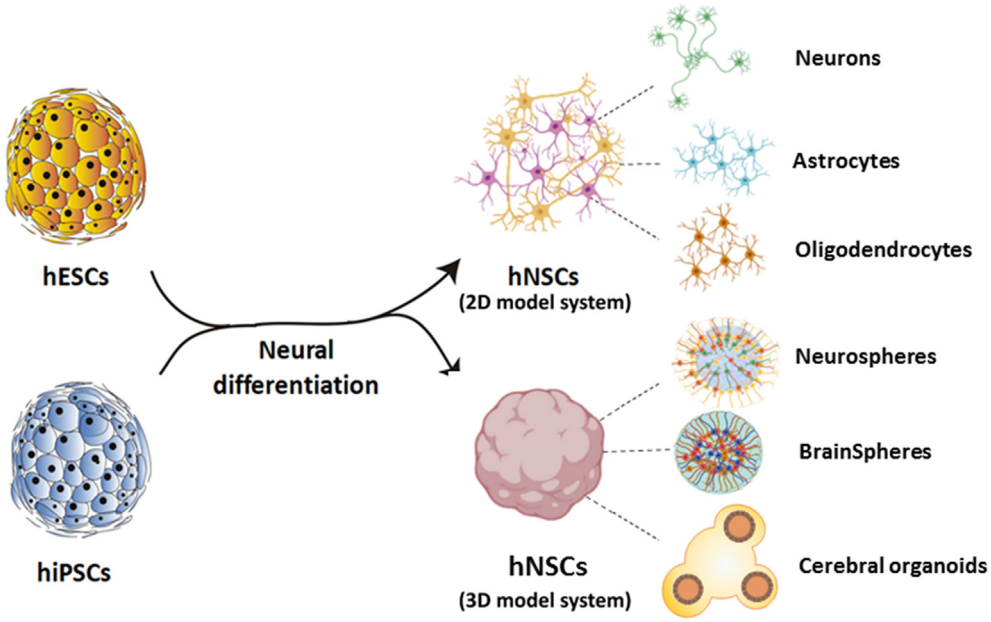

Figure 3. Human Pluripotent Stem Cells (hPSCs) (Human Embryonic Stem Cells (hESCs) and Human Induced Pluripotent Stem Cells (hiPSCs)) can be differentiated into 2D adherent Human Neural Stem Cells (hNSCs) or into 3D brain organoids.

4. 3D neural culture systems

2D model systems based on cell cultures derived from hPSCs consist of monolayer arranged cells, allowing their expansion and maintenance in a controlled environment (Chen et al., 2012b). In addition, they have the ability to recapitulate some of the biological processes that develop in the brain and, compared to classic *in vitro* models, allow the study of differentiation mechanisms, the processes involved in embryonic development, as well as the mechanisms involved in diseases (Pamies et al., 2017). However, the structural complexity and cellular heterogeneity of human brain has hindered, and limited in certain cases, by using only these 2D model systems (Lancaster et al., 2013). (Figure 3)

One of the advantages of using aggregates or 3D models is that they are cultures in suspension and therefore, compounds that bind to plastic can be washed out more effectively than in monolayer cell models (Harris et al., 2018).

Several studies have reported the applicability of human cell-based 3D models to assess cellular proliferation and differentiation, by using for example NT2 cells (Serra et al., 2007), that in presence of retinoic acid increase the differentiation and the amounts of neurons generated as compared to adherent cultures. In the same way, the immortalized cell line LUHMES, has been used in 3D cultures to analyze cellular toxicity, recovery and resilience after exposition to rotenone (Harris et al., 2018).

In recent years, several authors have raised the need for a 3D model system capable of recapitulating human brain development *in vitro* from hPSCs (Mariani et al., 2012; Lancaster et al., 2013; Paşca et al., 2015). These *in vitro* model systems are called "cerebral organoids or mini-brains" and are capable of mimicking brain morphogenesis and organogenesis (Fatehullah et al., 2016; Lancaster and Knoblich, 2014).

The human brain is characterized for its multiple neural (neurons, astrocytes, oligodendrocytes) and non-neuronal (microglia, blood vessels) cell types communicating with each other, these cell types do not exist in isolation. The development of brain organoids, has demonstrated a strong potential to reproduce the cellular and structural complexity of the prenatal developing brain, which cannot be captured in 2D monolayer culture systems (Lancaster and Knoblich, 2014). Furthermore, the human brain is highly complex in terms of neuronal projections and interactions. The development of the human brain begins around the third gestational week and follows a coordinated sequence of events that results in a spatially complex and regional diverse architecture. Cerebral organoids can contain multiple brain regions, including the cerebral cortex, choroid plexus, ganglionic eminence hippocampus and retina (Lancaster et al., 2013). This regional diversity may offer the opportunity to model the interaction of various brain regions.

The use of iPSCs in combination with the emerging human 3D tissue culture platforms, present a novel tool to predict and study human toxicity. By combining these technologies, multicellular brain spheroids (BrainSpheres) from hiPSC were generated. Rotenone (a commonly used plant pesticide) was then assessed to exert DNT which proved the model to be a reproducible and novel tool to study neurotoxicity and DNT. The authors demonstrated that rotenone treatment led to higher levels of reactive oxygen species, impaired neurite outgrowth and synaptogenesis (Pamies et al., 2018).

Yin and co-workers analysed the effects of exposure to the heavy metal cadmium during prenatal stages, for that they employed brain organoids which were engineered on array chips with octagon-shaped micro pillars, for studying neural dysfunctions. They observed increased cell death, impaired neurogenesis, and disturbed brain regionalization, suggesting impairment in the neurogenesis of fetal brain after cadmium exposure (Yin et al., 2018).

Zhong et al. (2020) used a 3D hiPSC-derived brain model (Brainspheres) to study the effects of an antidepressant, paroxetine, on key processes of brain development such as differentiation and maturation of brain cells, neurite outgrowth, synaptogenesis and myelination. Their results demonstrate that paroxetine is a potential human developmental neurotoxicant (Zhong et al., 2020).

In a more recent study, in order to screen larger sets of compounds with the potential for neurotoxicity, a hiPSC-based 3D neural platform composed of mature cortical neurons and astrocytes was employed. The 3D neural cultures presented spontaneous synchronized, readily detectable calcium oscillations. This neural platform was optimized for high-throughput screening in 384-well plates. Characterization of oscillation profiles in 3D neural cultures was performed through multi-parametric analysis. Cellular and mitochondrial toxicity were assessed by high-content imaging. For assay characterization, a set of neuromodulators with known mechanisms of action were used. The neurotoxic profile of a

library of 87 compounds (including pharmaceutical drugs, pesticides, flame retardants, and other chemicals) were finally explored. The results demonstrated that 57% of the tested compounds exhibited effects in the assay (Sirenko et al., 2019).

One of the limitations in the development of accurate organoids is the modelling of the microvasculature and BBB of the human cortex. To date, most *in vitro* BBB models utilize endothelial cells, pericytes and astrocytes; however, a recent study reports a 3D spheroid model of the BBB comprising all major cell types (including neurons, microglia and oligodendrocytes) which recapitulate more closely normal human brain tissue. Spheroids show expression of tight junctions, adherens junctions, adherens junction-associated proteins and cell specific markers. In addition, the BBB charge selectivity was assessed by evaluating inorganic mercury toxicity. For this functional assessment the use of MPTP, MPP+ and mercury chloride indicate charge selectivity through the barrier. These results indicate that the spheroid model may have potential applications in drug discovery, disease modelling, neurotoxicity and cytotoxicity testing (Nzou et al., 2018). In another interesting study (Schwartz et al., 2015) generated reproducible 3D neural constructs that incorporated vascular and microglial components for the detection of neurotoxicity using synthetic hydrogels under defined conditions. The authors managed to construct, from RNAseq data, a predictive model of toxicity using 60 toxic and non-toxic compounds.

5. Concluding remarks

The increase in neurodevelopmental diseases or disorders, together with the thousands of chemical substances and their mixtures, for which there is no information, requires the development of alternative methods for risk assessment in DNT, which are representative for humans and feasible from an ethical and economic point of view.

Human tumor cell lines could be an option to assess DNT, as alternative model of animal experimentation; however, alterations in the normal function of oncogenes could result in DNA damage that can give rise to accused differences between cell populations from one passage to another, and thus introducing great variability.

A promising alternative to the employment of the tumor lines can be the use of human stem cells. Stem cells provide an unlimited source of cells and can be kept in culture for long periods of time, and therefore can serve as chronic models of toxicity. In particular, special interest has been focused in hPSCs and more specifically hiPSCs.

Human iPSCs reproduce the "human context" *in vitro* by preserving the genetic and molecular phenotype of their donors and allow to address gene/environment interactions as well as the potential of chemicals to interfere with epigenetic mechanisms. In addition, hiPSCs can be guided to differentiate into cells of the CNS and combining them in a 2D or 3D format that allows to obtain complex models suitable for investigating neurotoxicity of brain-related diseases with patient-derived cells.

The generation of hiPSCs from adult somatic cells and the development 3D cultures of brain organoids have revolutionized the study of human brain development in both normal and pathological conditions.

The use of brain organoids could also be applied to assess the efficacy and toxicity of different drugs that would help detect safe and effective treatment options and could reduce the number of animals for the development of CNS therapeutics.

It is expected that the future *in vitro* 3D systems will become more complex, using multiple cell types to more faithfully represent the CNS or the brain (mixture with microglia, vessels, neurons, astrocytes, etc…).

These cultures provide a promising platform for analyzing/evaluating the effects of different pollutants or toxics during neurodevelopment process. As well as for *in vitro* testing of DNT and to generate valuable mechanistic data, speeding up the evaluation of compounds present in industrial, agricultural and consumer products that lack safety data on DNT potential.

Declarations

Author contribution statement

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Data availability statement

No data was used for the research described in the article.

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The authors declare no conflict of interest.

Additional information

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