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Review

An industry perspective: A streamlined screening strategy using alternative models for chemical assessment of developmental neurotoxicity

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ABSTRACT

Developmental neurotoxicity (DNT) is an important endpoint for the safety assessment of chemicals. However, the current *in vivo* animal model for DNT assessment is resource-intensive and may not fully capture all mechanisms that may be relevant to DNT in humans. As a result, there is a growing need for more reliable, time- and cost-efficient approaches for DNT evaluation. Toward this end, many stem/progenitor cell-based *in vitro* models and alternative organism-based models are becoming available with the potential for high throughput screening of DNT. Meanwhile, with advances in the knowledgebase of DNT molecular mechanisms and the identification of DNT-related adverse outcome pathways (AOP) there is potential to develop a mechanism-based integrated testing strategy for DNT assessment. This review summarizes the state of science regarding currently available human stem/progenitor cell-based *in vitro* models and alternative organism-based models that could be used for DNT testing. In addition, the current knowledge regarding DNT AOPs is reviewed to identify common key events that could serve as critical endpoints to assess multiple AOPs that underlie DNT. Following the identification of common key events, a streamlined strategy is proposed using alternative models to assess the DNT potential of chemicals as an early screening approach for chemicals in development.

1. Introduction

The developing brain is uniquely sensitive to environmental insults as it is critically dependent on precisely-orchestrated developmental processes that have to occur in a particular sequence at the right time and locations (Fritsche et al., 2018; Rice and Barone, 2000). The prevalence of diagnosed neurodevelopmental disorders is increasing worldwide and fuels the public concern regarding the lack of developmental neurotoxicity (DNT) data for a large number of chemicals (Bloom et al., 2012; Grandjean and Landrigan, 2006, 2014). Of the thousands of known chemicals, there is only a small fraction proven to cause DNT in humans (Fritsche et al., 2018; Grandjean and Landrigan, 2014). The paucity of DNT data is partially due to the fact that DNT evaluation is not a mandatory requirement unless triggered by evidence of developmental toxicity involving the nervous system, or neurotoxicity or endocrine disruption in systemic toxicity studies in adult rodents. There is potential that these triggers may not be sufficient as the adult brain lacks certain neurodevelopmental processes unique to the developing organism (Fritsche et al., 2018). In addition, conducting guideline-based DNT studies involves the use of large number of animals for an extended period of time; therefore, this study design is very resource intensive and unsuitable to screen large numbers of chemicals (Bal-Price et al., 2018b; Tsuji and Crofton, 2012). Furthermore, using rodent-based testing to predict DNT outcomes in humans has been criticized (Council, 2007) due to interspecies differences in molecular, cellular and functional characteristics (Baumann et al., 2016; Fritsche et al., 2018; Harrill et al., 2011). Therefore, there is an increasing demand for reliable, time- and cost-efficient approaches for DNT evaluation.

In line with this notion, many alternative methods have been developed to study human-specific DNT effects in a high throughput manner (Fritsche et al., 2015). Meanwhile, advances in the knowledgebase of DNT molecular mechanisms facilitates the identification of DNT adverse outcome pathways (AOP), which provide necessary

Abbreviations: DNT, developmental neurotoxicity; AOP, adverse outcome pathway; IATA, integrated approaches to testing and assessment; MIE, molecular initiating event; hESC, human embryonic stem cell; NPC, neural precursor cell; MeHg, methylmercury; MeHgCl, methylmercury chloride; CNS, central nervous system; MINC, migration inhibition of neural crest; MEA, microelectrode array; hiPSC, human induced pluripotent stem cell; HUCB-NSC, human umbilical cord blood-derived neural stem cell; NT2, human Ntera2/clone D1 embryocarcinoma cell; LUHMES, Lund human mesencephalic cell; GABA, gamma-amino butyric acid; KE, key event; AO, adverse outcome; CKE, common key event; NS/PC, neural stem/progenitor cell; T4, thyroxine

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information for developing mechanism-based integrated testing strategies for DNT assessment. Indeed, there are on-going international collaborative efforts that are focused on developing and validating integrated approaches to testing and assessment (IATA) frameworks that consist of large numbers of alternative DNT testing methods assessing a wide range of key neurodevelopmental events (Aschner et al., 2017; Bal-Price et al., 2018a,b; Fritsche et al., 2018). These IATA frameworks could provide broad range screening information regarding the DNT potential of chemicals; however, tremendous efforts are required to establish and validate numerous cell systems and assays that target multiple molecular initiating events (MIEs) and/or key neurodevelopmental events. As potential DNT activity of commercial/industrial products is only one toxicity of concern, there is an industry need for a DNT screening approach that is efficient and can detect a wide variety of potential modes of action when examining chemicals with unknown DNT potential. Therefore, numerous assays targeting different MIEs may not meet the needs of the chemical industry to efficiently screen chemicals for DNT potential during early stage of product development. In order to develop an efficient DNT testing strategy for industry, this article reviews currently available alternative models for DNT testing and the current knowledgebase of DNT AOPs. By identifying common key events that cover multiple AOPs and a set of assays that can be used to assess those key events, a streamlined screening strategy for DNT assessment is proposed.

2. Currently available alternative models for DNT assessment

The development and maturation of normal brain involves many critical neurodevelopmental processes that are potentially vulnerable to chemical exposure. These processes include neural progenitor cell proliferation, apoptosis, cell migration, neuronal and glial differentiation, neurite outgrowth and branching, myelination, synaptogenesis and neuronal network formation (Rice and Barone, 2000; Stiles and Jernigan, 2010; Tau and Peterson, 2010). Increasing numbers of *in vitro* and non-mammalian organism models are being developed to evaluate those key neurodevelopmental processes to infer DNT potential of chemicals. The following sections will discuss those models that have been used in testing the effects of chemicals on neurodevelopment and will focus on human stem/progenitor cell-based models (both in 2D and 3D culture) and non-mammalian organism-based models. The selection of studies does not purport to be complete, but spans a good representation of currently available options.

2.1. Human stem/progenitor cell-based 2D models

2.1.1. Human embryonic stem cells (hESC)

The embryonic stem cells possess the ability of *in vitro* differentiation into cell types of all three germ layers, which to some extent reflect the human physiology of embryogenesis. Therefore, hESC differentiation has been recognized as a promising tool for studying developmental neurotoxicity (Stummann and Bremer, 2008). The hESC-based *in vitro* neurodevelopmental process typically involves four steps: (1) embryonic body formation in hESC culture; (2) the generation of neural-tube like rosettes, which consist of columnar epithelial cells and represent the early developing neuroepithelium. The rosette is considered as the source of neural precursor cells (NPC); (3) NPC proliferation; and (4) the differentiation of NPCs into neurons, astrocytes and oligodendrocytes (Li et al., 2005; Perrier et al., 2004; Zhang et al., 2001). A growing number of studies have utilized this *in vitro* neuronal development model to evaluate DNT potential of chemicals by measuring a wide range of endpoints, which includes cell differentiation, proliferation, apoptosis, migration, neurite outgrowth and neuronal network formation.

2.1.1.1. Differentiation. The differentiation of hESCs to neurons and glia cells is considered to be a vulnerable process that is subject to disturbance by toxic insults that results in deficits in the nervous system (Rice and Barone, 2000). The alterations of neural differentiation are primarily assessed by gene expression and rosette morphometry. Stummann et al. studied the effects on neuron differentiation by methylmercury (MeHg), a well-documented developmental neurotoxicant (Stummann et al., 2009). They found MeHg treatment altered the expression of several neuronal mRNA markers at non-cytotoxic concentrations during hESCs to NPC differentiation, but not in NPC maturation, indicating that NPC generation is more sensitive to MeHg than later stages of neuronal differentiation (Stummann et al., 2009). Likewise, Colleoni et al. (2011, 2012) found that retinoic acid and valproic acid, well-known teratogenic agents, altered the morphology of hESC-derived rosettes as well as the gene expression that is relevant to early neural development. Additionally, Talens-Visconti et al. (2011) demonstrated that ethanol decreased the proliferation of NPC cells and reduced their differentiation into mature neurons, astrocytes, and oligodendrocytes. The ethanol-mediated decrease in astrocyte differentiation was also observed in another study (Nash et al., 2012), whereas an increase in hESC and NPC proliferation was reported.

2.1.1.2. Migration. In addition to the neural cell types that comprise the central nervous system (CNS), hESC-derived neural rosettes can also give rise to neural crest cells (Chambers et al., 2009; Lee et al., 2010; Lee et al., 2007). During vertebrate embryogenesis, neural crest cells emerge within the dorsal margins of the closing neural folds, and migrate extensively to several different locations in the embryo to generate a variety of cell lineages, including peripheral neurons and glia (Achilleos and Trainor, 2012). Therefore, the migration capacity of neural crest cells is essential for the peripheral nervous system development (Dupin and Sommer, 2012). There are several toxicological studies measuring the migration of hESC-derived neural crest cells and/or the expression of migration-related genes to evaluate the DNT potential of chemicals. Zimmer et al. developed a 'migration inhibition of neural crest' (MINC) assay that quantifies the migration of neural crest cells into a pipette tip scratched cell-free gap (Zimmer et al., 2012). The performance of the MINC assay was evaluated by treatment with several known neurodevelopmental toxicants: valproic acid, methylmercury, and lead. A subsequent study employed the MINC assay to test the developmental toxicity of a wide range of compounds including several biologics, classical pharmaceuticals and also six environmental toxicants (Zimmer et al., 2014). This effort was a component of the European Union research consortium for the use of 'embryonic stem cell-based novel alternative tests' for the prediction of toxicity of drug candidates. In addition, Dreser et al. (2015) has also demonstrated the disturbance of neural crest cell migration by 16 environmental toxicants that belong to diverse groups, including heavy metals, polychlorinated biphenyls, pesticides, and histone deacetylase inhibitors. They also analyzed the expression changes of 35 migration-related genes after toxicants treatment and showed that mechanistically related compounds triggered similar transcriptional changes. Furthermore, transcriptomic data were utilized to identify transcription signatures and biomarkers specific for potential developmental toxicants that inhibit human neural crest cell migration (Pallocca et al., 2016).

2.1.1.3. Apoptosis. The proper development of the nervous system requires apoptosis that systematically removes neurons in some structures generated during ontogeny, therefore perturbation of apoptosis by neurotoxic agents might result in altered cell number and neural function (Rice and Barone, 2000). To this end, a variety of apoptotic assays were used to assess chemical-induced alterations of cell apoptosis during hESC to neuronal cell differentiation. Lee et al. (2014) studied the developmental neurotoxicity of chlorpyrifos, one of the most widely used organophosphate insecticides with known neurotoxicity. The treatment of chlorpyrifos on hESC-derived NPCs

led to a decrease in cell viability. Concomitantly, chlorpyrifos treatment increased production of reactive oxygen species, cytochrome c release, and activation of caspase-9 and -3, indicating that cell death induced by chlorpyrifos was due to apoptosis in hNPCs. Likewise, Ketamine, a widely used pediatric anesthetic, has also been reported to increase cytochrome c release and mitochondrial fission in hESC-derived neurons and lead to neuronal apoptosis (Bai et al., 2013). Moreover, Zeng et al. (2006) demonstrated that 1-methyl-4-phenylpyridinium induced the formation of intracellular reactive oxygen species and apoptotic cell death of hESC-derived dopaminergic neurons. Although chemical-induced apoptosis in neurons implicates the potential adverse effect of chemicals, additional work will be needed to confirm the DNT potential of those chemicals by demonstrating the specificity of those apoptotic effects to developing neurons and/or by performing comparative dosimetry to show the relative sensitivity of developing neurons to the apoptotic effects.

2.1.1.4. Neurite outgrowth and branching. During neuronal differentiation, newly generated neurons undergo extensive morphological changes including emergence of neurites, neurite outgrowth and branching. These morphological changes are critical for establishment of cell–cell contacts and the formation of the intricate neural networks that facilitate nervous system function (Munno and Syed, 2003). The disruptions of these events during developmental stages by neurotoxic compounds could potentially result in deficits in nervous system function in later life stages (Costa et al., 2004; Grandjean and Landrigan, 2006). The assessment of neurite outgrowth and branching are generally conducted *via* cell imaging by quantifying parameters including average number of neurites per neuron, total neurite length per neuron and the extent of neurite branching. One *in vitro* approach reported by Harrill et al. (2010) quantified neurite outgrowth in human embryonic stem cell-derived hN2 cells using automated high-content image analysis. In this study, concentration-dependent decreases in neurite outgrowth and ATP-content in hN2 cells were observed following treatment with chemicals that have previously been shown to inhibit neurite outgrowth in primary rodent neural cultures, including bisindolylmaleimide I, U0126, lithium chloride, sodium orthovanadate and brefeldin A. A subsequent study compared sensitivity of human hN2 cells and rat primary neural cultures to chemical-induced inhibition of neurite outgrowth (Harrill et al., 2011). They found that although human hN2 cells had a lower dynamic range for detecting chemical-induced neurite outgrowth inhibition and greater variability as compared to rat primary cortical cultures, human neural cultures were more sensitive to a set of chemicals previously shown to inhibit neurite outgrowth. Likewise, He et al. studied the effects of MeHg exposure on the morphology of neurons derived from mouse and human embryonic stem cells (He et al., 2012). They demonstrated that the neurite length and branch points of ESC-derived neurons were more susceptible to a low concentration of MeHg in human than in mouse. In addition, Hoelting et al. adopted a two-step protocol to generate peripheral human neuronal precursors and subsequent dorsal root ganglia neurons (Hoelting et al., 2016). Neurite growth and cell viability were quantified using high content imaging to assess neurotoxicity of 35 chemicals. The performance of the testing assay was validated by using known neurotoxicants and neurite growth enhancers. Interestingly, various classes of chemotherapeutic agents causing human peripheral neuropathies were identified in this peripheral neurotoxicity test, and they were missed when tested on human central neurons (LUHMES dopaminergic neuronal precursor cells), demonstrating the specificity of this assay.

2.1.1.5. Neuronal network formation. Lastly, the formation of a functional neuronal network is critical for nervous system development and function. It has been demonstrated that the spatiotemporal pattern of the neuronal network activity can be robustly measured *in vitro* by measuring electrophysiological conductance using microelectrode arrays (MEA) (the utility of MEA techniques in neurotoxicity testing has been reviewed by Johnstone et al. (2010)). The ability of MEA to detect perturbations in spontaneous neuronal network activities was characterized and validated using well-characterized training set chemicals (Brown et al., 2016; Cotterill et al., 2016; Mack et al., 2014; Vassallo et al., 2017). Moreover, large libraries of chemicals (*e.g.* ToxCast libraries) have been screened for alterations in neuronal network function in a high-throughput fashion using multi-well MEA plates (Frank et al., 2018; Strickland et al., 2018; Valdivia et al., 2014). Although the aforementioned studies were carried out mainly in rat primary cortical cells, advances have been made to use hESC-derived neurons on MEA to detect alterations in neuronal network activity by chemicals, thereby circumventing the interspecies extrapolation (Heikkila et al., 2009; Kapucu et al., 2012; Yla-Outinen et al., 2010). Heikkila et al. (2009) demonstrated that hESC-derived neuronal cells can form spontaneously active networks with synchronous communication *in vitro*, and are therefore suitable for use in DNT screening studies. In addition, Yla-Outinen et al. (2010) utilized MEA to study the neurotoxicity of MeHg chloride (MeHgCl) on a hESC-derived neuronal network. This study showed that the electrical activities of hESC-derived neuronal network were altered by MeHgCl at a concentration that did not produce detectable effects on cell proliferation, cell survival, and gene and protein expression, suggesting that the alteration in neuronal network activity is a sensitive endpoint for evaluating DNT.

Taken together, hESC-based models are capable of assessing effects of chemicals on the whole process of neural development from hESC to NPC and ultimately to neurons and glia cells. There are a wide variety of endpoints (*e.g.*, proliferation, differentiation, apoptosis, migration, neurite outgrowth and neuronal network activity) that can be assessed in various cell types at different developmental stages, which provide wide-ranging information for DNT evaluation. However, the use of hESC requires special permission due to ethical concerns, which limits the utility of hESC for DNT testing.

2.1.2. Human induced pluripotent stem cell (hiPSC)

Since the use of hESCs raises ethical concerns, hiPSC is considered as an alternative cell type that allows *in vitro* neuronal development and can serve as a model for studying DNT. A growing body of data in the literature has demonstrated that hiPSC is capable of developing into neural stem cells, neurons, oligodendrocyte and astrocytes (Hu et al., 2010; Liu et al., 2013; Shaltouki et al., 2013; Swistowski et al., 2010; Wang et al., 2013). The disturbance of neurodevelopmental processes initiated from hiPSCs has been measured as a readout to evaluate DNT potential of chemicals using similar endpoints as described for hESCs. For instance, a number of studies have examined the relative sensitivity of developing neuronal and/or glial cell types to toxicant-induced cytotoxicity. Pei et al. (2016) have assessed a 80-compound library comprised of drugs (*e.g.*, valproic acid) and pesticides (*e.g.*, aldicarb, rotenone) with known neurotoxic potential as well as environmental compounds with unknown neurotoxic potential (*e.g.*, flame retardants, polycyclic aromatic hydrocarbons) for their cytotoxic effect on iPSC as well as iPSC-derived neural stem cells, neurons, and astrocytes using the MTT assay. After 24-h treatment at 10 and 100 μM, 40% to 58% of the compounds induced significant cytotoxicity in the four cell types with cell-type specificity. In addition, the cytotoxicity of selected compounds were confirmed using a simplified assay that quantifies luciferase activity in lineage-specific luciferase reporter iPSC lines that were generated previously (Pei et al., 2015). Likewise, Malik et al. (2014) screened a library of 2000 compounds for cytotoxicity on human iPSC-derived neural stem cells, neurons and rat cortical cells. They identified approximately 100 of the tested compounds showed specific toxicity to human neural stem cells. A follow-up screen of 43 compounds on human iPSCs, iPSC-derived neurons, and fetal astrocytes indicated a validation rate of > 80%.

In addition to cytotoxicity, high-throughput assays for characterizing neurite morphology of hiPSC-derived neuronal cells were developed using high-content imaging (Sirenko et al., 2014), which could be used for DNT assessment. For instance, Wheeler et al. studied neurotoxicity of chemotherapeutics (*e.g.*, paclitaxel, vincristine or cisplatin) using hiPSC-derived neuronal cells from four individuals (Wheeler et al., 2015). Consistent and reproducible decreases in total neurite outgrowth, number of processes and branches, and cell viability were observed after drug treatment. Despite of the overall consistency, the authors found that the variance in several neurite morphology and apoptotic phenotypes upon treatment was greater between individuals than within individuals. This result raises the awareness that genetic diversity among human population could influence the interpretation of the human cell-based DNT assessments and should be taken into consideration in safety assessment. Currently, although the genetic diversity required to make iPSC-derived neurons/glia a representative model has not been determined, iPSCs derived from somatic cells of multiple donors has the potential to be a genetically diverse model for DNT assessment.

Recently, the neuronal network activity of hiPSC-derived cultures has been characterized using MEA (Tukker et al., 2016). In this study, the composition of hiPSC-derived neuronal cultures from various suppliers was assessed using immunofluorescent staining. Mixed cell populations were identified in these cultures that consist of excitatory neurons, inhibitory neurons and astrocytes. Using multi-well MEA, the authors demonstrated that these hiPSC-derived cultures develop spontaneous neuronal activity over time. Furthermore, the modulation of neuronal activities by different physiological, toxicological and pharmacological compounds were observed, suggesting the suitability of these hiPSC-derived neuronal cultures for *in vitro* DNT screening.

Overall, hiPSC has been demonstrated to have the capability for *in vitro* neurodevelopment, and therefore, could serve as a source of stem cells for DNT testing without ethical controversy. Like hESC, hiPSC models can be used to assess multiple endpoints in a variety of neural cell lineages to evaluate the DNT potential of chemicals. Additionally, since hiPSCs are derived from reprogrammed somatic cells (Takahashi et al., 2007), it is possible to generate genetically diverse hiPSC models and to predict DNT in genetically susceptible populations (Habela et al., 2016; Ohara et al., 2017).

2.1.3. Human umbilical cord blood-derived neural stem cell (HUCB-NSC)

HUCB-NSC is a non-transformed, self-renewing neural stem/progenitor cell line isolated and established from human umbilical cord blood (Bużańska et al., 2002). HUCB-NSC has been demonstrated to maintain the differentiating potential into neuronal, astroglial, or oligodendroglial cells (Buzanska et al., 2005; Bużańska et al., 2002). A subsequent study has assessed the sensitivity of HUCB-NSCs at different developmental stages to a panel of neurotoxic (*e.g.*, odium tellurite, MeHgCl, cadmium chloride, chlorpyrifos, and L-glutamate) and non-neurotoxic (*e.g.*, acetaminophen, theophylline, and D-glutamate) compounds (Buzanska et al., 2009). Key neurodevelopmental processes were examined, including cell proliferation, apoptosis, and neuronal and glial differentiation. Investigators demonstrated the ability of the HUCB-NSC model to discriminate between neurotoxic and non-neurotoxic compounds and found that the susceptibility of HUCB-NSCs to some compounds was developmental stage and cell type dependent, suggesting the usefulness of the HUCB-NSC model for DNT testing.

The HUCB-NSC line is clonal and karyotypically stable and has been shown to grow in culture for more than 45 passages over 3 years (Buzanska et al., 2005), representing an unlimited and ethically uncontroversial source of neural stem cells. Coupling with the neural differentiation potential, HUCB-NSC appears to be a promising model for *in vitro* DNT testing. However, it is currently unclear how the cord blood-derived HUCB-NSCs are similar to primary neural stem cells. Comprehensive comparisons are needed to clarify this uncertainty.

2.1.4. Human Ntera2/clone D1 (NT2) embryocarcinoma cell

NT2 cells are a human pluripotent embryonic carcinoma cell line and has been demonstrated to be able to differentiate into neuronal and glial cells after 7 weeks of culture *in vitro* (Laurenza et al., 2013). In addition, spontaneous electrical activity of the NT2-derived neurons was observed *via* MEA assays, indicating the maturation of the neuronal network. A treatment of this NT2-derived neuronal/glial culture with MeHg, lead or aluminum revealed alterations in expression of neuronal and glial cell-specific genes. In another study, Stern et al. (2014) established high-throughput assays to assess the DNT potential of chemicals using the human NT2 cell line. The disturbance of neuronal differentiation and migration was assessed using a standard fluorescence plate reader, enabling high-throughput screening. To demonstrate the suitability of this model for DNT studies, developing NT2 cells were treated with four test compounds with well-established DNT potential: MeHgCl, sodium arsenite, sodium valproate, and methylazoxymethanol. This study showed that these compounds significantly reduced the expression of the neuronal marker beta-tubulin type III and decreased the migration distance in developing NT2 cells.

Although NT2 cells represent an unlimited source of stem cells and could serve as an alternative to primary pluripotent stem cells, the ability of the NT2 model to assess effects on neuronal cell proliferation and apoptosis is called into question due to the immortal nature of the cell line. Further characterization of the NT2 cells and comparison with primary cells would help to mitigate this concern.

2.1.5. Lund human mesencephalic (LUHMES) cells

LUHMES cells are an immortalized cell line generated by overexpressing v-myc in fetal human mesencephalic cells (Lotharius and Brundin, 2002). LUHMES cells can be differentiated into morphologically and biochemically mature dopamine-like neurons that exhibit dopaminergic and neuronal characteristics (Lotharius et al., 2005; Scholz et al., 2011). Studies have assessed chemical-induced impairment of neurite outgrowth of LUHMES cells (Krug et al., 2013; Stiegler et al., 2011). Researchers found that neurite extension of developing LUHMES was specifically inhibited by diverse compounds such as U0126, colchicine, vincristine, narciclasine, rotenone, cycloheximide, or diquat. These compounds reduced neurite growth at concentrations that did not compromise cell viability, as evidenced by the comparison of the EC50 values of neurite growth inhibition and cell death resulting in a ratio greater than 4. On the contrary, unspecific cytotoxicants such as menadione, cadmium chloride, and sodium dodecyl sulfate yielded ratios less than 4. These studies suggested the ratio of EC50 (neurites)/EC50 (cell death) along with an arbitrary cut-off provided a robust classifier for compounds associated with a developmental neurotoxic hazard.

Although neuronal properties of LUHMES-derived dopaminergic neurons have been characterized, including the expression of neuronal markers, neurite outgrowth and electrophysiological properties (Scholz et al., 2011), the ectopic expression of v-myc raises the concern that LUHMES cells may not fully recapitulate neuronal differentiation *in vivo*. Comprehensive comparisons between LUHMES and primary cells for their responsiveness to a large set of well-characterized neurotoxicants and nontoxic control chemicals would help to mitigate this concern. In addition, *in vitro* differentiation of LUHMES generates homogenous dopaminergic neurons without astroglia or oligodendroglia; therefore, further development of the model by incorporating glia cells would better mimic the physiological microenvironment of the CNS.

2.1.6. ReNcell

ReNcell CX cells are an immortalized neural progenitor cell line derived from the cortex of a 14-week human fetus that have been demonstrated to be able to proliferate and differentiate into neuronal, astrocytic, and oligodendrocytic cell populations (Donato et al., 2007). Breier et al. (2008) developed and optimized a high-throughput

screening assay for chemical effects on the proliferation and viability of ReNcell CX cells using high-content imaging. A subsequent study investigated the effects of 11 chemicals (cadmium, chlorpyrifos oxon, dexamethasone, dieldrin, ketamine, lead, maneb, MeHg, nicotine, trans-retinoic acid, and trimethyltin) on proliferation and apoptosis of neural progenitor cells (Culbreth et al., 2012). Researchers compared chemical-induced changes in human ReNcell CX cells and mouse neural progenitor cells and found human cells were more sensitive than mouse cells for the cell proliferation endpoint. Recently, the ReNcell CX cell-based proliferation/cytotoxicity assays were also used to screen the ToxCast Phase I library of 309 chemicals (Radio et al., 2015).

The aforementioned studies have demonstrated the usefulness of human ReNcell CX cells to assess the effects of chemicals on proliferation and survival of human neural progenitor cells in a high-throughput fashion. The utility of ReNcell CX cells for other DNT related endpoints (*e.g.*, neural differentiation, migration and neuronal network formation) is yet to be explored.

2.2. Human stem/progenitor cell-based 3D models

There is growing awareness that 3D culture models may better mimic the *in vivo* cellular microenvironment and cellular interactions during neural development, and therefore may be more physiologically relevant for DNT testing. Here we review the studies that assess the DNT potential of chemicals using the emerging 3D culture techniques.

2.2.1. hESC-derived 3D neural constructs

Hoelting et al. (2013) have developed a 3D *in vitro* model derived from hESCs to evaluate the DNT potential of chemicals by monitoring neurodevelopmental gene expression. First, the neuronal differentiation within the 3D model was characterized by various neural markers and gene expression profiling. The responsiveness of the 3D model to a known developmental neurotoxicant was confirmed by alterations of neural gene expression due to exposure to non-cytotoxic concentrations of MeHg. Subsequent tests for the specific DNT of polyethylene nanoparticles were performed and found that these nanoparticles penetrated deep into the 3-D structures and altered gene expression, including NOTCH pathway genes and downstream neuronal precursor genes. Furthermore, Schwartz et al. (2015) developed a complex self-assembled 3D neural construct by combining hESC-derived neural progenitor cells, endothelial cells, mesenchymal stem cells, and microglia/macrophage precursors to model cellular interactions within the developing brain. The 3D neural constructs have been demonstrated to comprise diverse neuronal and glial populations, interconnected vascular networks, and microglia. The reproducibility of 3D neural constructs were validated by RNA sequencing. Then, machine learning was used to build a predictive model from changes in global gene expression of 240 neural constructs exposed to 34 toxic and 26 nontoxic training chemicals. Subsequently, this predictive model correctly classified 9 of 10 additional chemicals in a blinded trial, demonstrating the value of this model for DNT assessment. In addition, Sandstrom et al. (2017) developed a protocol for generating 3D human neural tissues using neural progenitor cells derived from hESCs. Progressive neuronal and glial differentiation as well as myelination were observed. Functionality of the neural tissue was also evaluated with electrophysiological recordings. Subsequently, the effects of an acute exposure to three known neurotoxicants (MeHg, trimethyl tin and paraquat) were assessed by measuring alterations in gene expression (Sandstrom et al., 2017). Moreover, Mayer et al. reported a hESC-derived 3D neurosphere model and characterized electrophysiological properties of neurospheres using MEA (Mayer et al., 2018). In this study, spontaneous neuronal network activities were detected within neurospheres over time, which could be further enhanced and showed a synchronous pattern by treatment with a gamma-amino butyric acid (GABA) receptor antagonist.

The aforementioned studies demonstrate the great potential of using hESC-derived 3D neural constructs in DNT assessment. Currently, the reported endpoints for DNT assessment were primarily focused on gene expression. It is anticipated that more and more studies will use these emerging models to assess DNT potential based on a wide range of critical endpoints (*e.g.*, cell viability, neuronal and glial differentiation and neuronal network, *etc.*). The resulting growing database could be used to further characterize the applicability and performance of these models in DNT testing.

2.2.2. iPSC-derived human 3D brain microphysiological system

Recently, an iPSC-derived human 3D brain microphysiological system was developed, which comprised differentiated mature neurons (glutamatergic, dopaminergic and GABAergic neurons) and glial cells (astrocytes and oligodendrocytes) (Pamies et al., 2017). The reproducibility of the culture system was validated by quantification of the different cell types within the system. More importantly, the model has been demonstrated to reproduce neuronal-glial interactions and connectivity, such as synaptogenesis, spontaneous electrical activity and axon myelination. After 8 weeks of differentiation, the 3D microphysiological system showed 40% overall myelination quantified by immunohistochemistry and confirmed by confocal microscopy and electron microscopy. The detection of myelination represents a unique applicability of this model to assess the effects of chemicals on oligodendroglial function and myelination during brain development.

Although the iPSC-derived human 3D brain microphysiological system holds great potential to test DNT of chemicals on a variety of endpoints (*e.g.*, cell viability, neuronal differentiation, glial differentiation, synaptogenesis, neuronal network function and myelination), validation for the sensitivity and specificity of the model by using a set of well-characterized training chemicals is yet to be conducted.

2.2.3. Human NT2 embryocarcinoma cell-derived neurospheres

The differentiation of NT2 cells into mature neurons in 2D platform is a lengthy process, which typically takes 6–7 weeks (Andrews, 1984). Further advancement has demonstrated that NT2 cells grown into 3D neurospheres can generate neurons and astrocytes within 14 days of culture, which makes it an efficient model system for DNT testing (Hill et al., 2008). In this study, NT2 cell-derived neurospheres were exposed to known human teratogens, including non-embryotoxic (acrylamide), weakly embryotoxic (lithium, valproic acid) and strongly embryotoxic (hydroxyurea) chemicals. Cell viability and neuronal protein marker expression were examined to identify developmental neurotoxicants. Differential effects were observed in NT2 cell-derived neurospheres in response to different teratogens, suggesting this model could be used for *in vitro* DNT testing. More recently, Terrasso et al. (2015) developed a scalable bioprocess for neural differentiation of human NT2 cells in stirred culture systems. Endpoints based on neuronal- and astrocytic-specific gene expression were assessed in multi-well format. Alterations in the expression of the presynaptic and cytoskeleton markers were observed when NT2 cell-derived neurospheres were exposed to the neurotoxicants acrylamide and chloramphenicol, respectively. This 3D approach allowed efficient production of large numbers of human differentiated neurospheres and could serve as a cell model to evaluate human neuronal and astrocytic toxicity. However, the immortal nature of the cell line might cast some uncertainty over its ability to reliably assess effects on neuronal cell proliferation and apoptosis (Fritsche, 2017). Further comparison with primary cells may help to mitigate these concerns.

2.2.4. Neurospheres derived from primary human NPCs

Neurospheres derived from primary human NPCs have been demonstrated to imitate some of the key processes of human neuronal development, including proliferation, migration, and differentiation into neurons and glial cells (Baumann et al., 2016; Fritsche et al., 2005; Moors et al., 2009; Schreiber et al., 2010). By using this neurosphere model, Moors et al. showed that the developmental neurotoxicants MeHgCl and HgCl decreased migration distance of NPCs and number of

Table 1
Summary of human stem/progenitor cell-base models.

Models	Cell types	End points	References
2D models	hESC	Differentiation	Stummann et al., 2009; Colleoni et al., 2011, 2012; Talens-Visconti et al., 2011; Nash et al., 2012
		Migration	Zimmer et al., 2012, 2014; Dreser et al., 2015; Pallocca et al., 2016
		Viability	Lee et al., 2014; Bai et al., 2013; Zeng et al., 2006
		Neurite outgrowth	Harrill et al., 2010, 2011; He et al., 2012; Hoelting et al., 2016
		Neuronal network formation	Heikkila et al., 2009; Kapucu et al., 2012; Yla-Outinen et al., 2010
	hiPSC	Differentiation	Malik et al., 2014; Pei et al., 2015
		Viability	Malik et al., 2014; Pei et al., 2015
		Neurite outgrowth	Wheeler et al., 2015
		Neuronal network formation	Tukker et al., 2016
	HUCB-NSC	Proliferation	Buzanska et al., 2009
		Differentiation	Buzanska et al., 2009
		Viability	Buzanska et al., 2009
	NT2	Differentiation	Laurenza et al., 2013; Stern et al., 2014
		Migration	Stern et al., 2014
	LUHMES	Viability	Krug et al., 2013; Stiegler et al., 2011
		Neurite outgrowth	Krug et al., 2013; Stiegler et al., 2011
	ReNcell	Proliferation	Breier et al., 2008; Radio et al., 2015
		Viability	Breier et al., 2008; Radio et al., 2015
3D models	hESC	Differentiation	Hoelting et al., 2013; Schwartz et al., 2015; Sandstrom et al., 2017
		Viability	Hoelting et al., 2013; Sandstrom et al., 2017
		Neuronal network formation	Sandstrom et al., 2017; Mayer et al., 2018
	iPSC	Differentiation	Pamies et al., 2017
		Myelination	Pamies et al., 2017
		Neuronal network formation	Pamies et al., 2017
	NT2	Differentiation	Hill et al., 2008; Terrasso et al., 2015
		Viability	Hill et al., 2008
	Primary human NPCs	Viability	Moors et al., 2009; Baumann et al., 2016
		Proliferation	Baumann et al., 2016
		Migration	Moors et al., 2009; Schreiber et al., 2010; Barenys et al., 2017; Baumann et al., 2016
		Differentiation	Schreiber et al., 2010; Baumann et al., 2016
	ReNcell VM	Viability	Meli et al., 2014; Nierode et al., 2016
		Differentiation	Meli et al., 2014
	LUHMES	Viability	Smirnova et al., 2016
		Differentiation	Smirnova et al., 2016

This table summarized the examples of human stem/progenitor cell-base *in vitro* models that have been used in testing the effects of chemicals on neurodevelopment. The selection of studies does not purport to be complete.

neuronal cells that were derived from NPCs (Moors et al., 2009). Furthermore, NPC apoptosis was also observed when exposed to high amounts of oxidative stress. Likewise, Schreiber et al. demonstrated that polybrominated diphenyl ether flame retardants decreased migration distance of NPCs and reduced the differentiation of NPCs into neurons and oligodendrocytes (Schreiber et al., 2010). They also found that simultaneous exposure with the thyroid hormone receptor agonist triiodothyronine rescued these effects on migration and differentiation, suggesting that polybrominated diphenyl ethers disturb the *in vitro* development of human NPCs *via* disruption of cellular thyroid hormone signaling. More recently, Barenys et al. (2017) reported that the green tea catechin, epigallocatechin gallate, altered human and rat neural development *in vitro* by inhibiting adhesion and migration of NPCs, raising a concern for the DNT potential of epigallocatechin gallate and highlighting the need for *in vivo* characterization. To investigate species differences in chemical effects on neurodevelopmental key events, Baumann et al. (2016) compared the responses of human and rat neurospheres to a training set of nine chemicals (positive substances: MeHgCl, sodium metaarsenite, methylazoxy methanol acetate, valproic acid, chlorpyrifos ethyl, parathion ethyl; and negative substances: glutamate, paracetamol, penicillin G sodium salt). They found that human and rat neurospheres showed different sensitivity to most chemicals, suggesting species differences in toxicodynamics of chemicals. Moreover, human and rat neurosphere assays classified most chemicals correctly, highlighting the potential use of neurospheres as a component of an *in vitro* DNT testing strategy.

The limitations for using neurosphere assays in high-throughput screening include the time-consuming handling of single neurospheres and the difficulties in phenotypic assessments of NPC migration and differentiation. Recently, efforts have been made to overcome these obstacles by employing automated neurosphere handling and high-content image analysis approaches (Gassmann et al., 2012; Schmuck et al., 2017). The use of primary human NPC-derived neurosphere for DNT testing is also limited due to the availability issues and ethical concerns, therefore, 3D models using human neural stem/progenitor cell lines as alternative cell sources are being developed for *in vitro* DNT screening.

2.2.5. Neural development 3D models using human neural stem/progenitor cell lines

ReNcell VM is an immortalized human neural stem cell line derived from the ventral mesencephalon region of a 10-week human fetal brain. ReNcell VM cells maintain a stable normal karyotype and can be differentiated into neurons (primarily dopaminergic cells), astrocytes, and oligodendrocytes (Donato et al., 2007). Meli et al. (2014) developed a 3D cellular microarray platform for the high-throughput analysis of human neural stem cell growth and differentiation by culturing ReNcell VM on a miniaturized cell culture chip consisting of 60 nL spots of cells encapsulated in alginate. The viability and differentiation of neural stem cells were assessed by a live/dead cell viability assay and an on-

chip cellular immunofluorescence assay. Using this 3D cellular microarray culture system, cell stage-dependent responses of ReNcell VM cells to a set of neurotoxicants (5-fluorouracil, cadmium chloride, dexamethasone and retinoic acid) were observed. A subsequent study employed the 3D cell culture chip for high-throughput screening and identified 24 compounds with differential toxicity on undifferentiated and differentiating ReNcell VM cells, demonstrating this platform has potential use in high throughput predictive DNT screening (Nierode et al., 2016).

As mentioned earlier, LUHMES cell line is derived from human fetal mesencephalic cells by overexpressing v-myc. Smirnova et al. (2016) developed a LUHMES 3D model that allows for an extended period of culture. A highly reproducible differentiation process was observed in this 3D model using confocal microscopy, gene expression, and flow cytometry. The expression of genes and miRNAs known to be involved in mitochondrial function were altered in response to two model neurotoxic compounds, rotenone and MPP+, demonstrating the suitability of this 3D model for neurotoxicity testing.

Summary of human stem/progenitor cell-based 3D models

Overall, the aforementioned newly emerging 3D models hold great potential for DNT testing as the 3D architecture is an advantage for the cell-cell interactions that are important for neuronal development and hence these models are inherently more similar to *in vivo* physiology (Stoppini et al., 2017). In spite of this advantage, some of the human cell-based 3D models, especially the newly emerging ones, have not been widely adopted and tested across many different labs, therefore the inter-laboratory reproducibility and robustness of these models have not been fully evaluated (Bal-Price et al., 2018a; Fritsche, 2017).

Summary of human stem/progenitor cell-based *in vitro* models

Taken together, human stem/progenitor cell based *in vitro* models (both 2D and 3D, summarized in Table 1) represent promising alternatives to rodent models for DNT evaluation due to the advantage of circumventing interspecies extrapolation. Moreover, *in vitro* models enable high-throughput screening and are therefore time- and cost-efficient. The readiness of *in vitro* models has been discussed in a recent review by Bal-Price et al. (2018a). On the other hand, there are limitations to *in vitro* models to assess DNT, including: (1) lacking toxicokinetics (*e.g.*, limited metabolic capacity); (2) cannot capture the effects of chemical on other organ systems (*e.g.*, endocrine system) that either subsequently lead to deficits in nervous system development or drive toxicity reference doses before DNT effects occur; and (3) cannot evaluate behavioral and cognitive functions. These shortcomings can potentially be alleviated by coupling *in vitro* testing with organism-based models, and subsequently rodent models for higher level functions.

2.3. Non-mammalian organism-based models

In light of refining animal use, the potential of using alternative non-mammalian species for DNT testing has been explored. Studies have demonstrated that many of the basic molecular developmental and functional aspects of the nervous system are conserved across mammalian and non-mammalian alternative species, including zebrafish and *C. elegans*. During the past decade, zebrafish and *C. elegans* have been increasingly used as models for neurotoxicity (see reviews (Avila et al., 2012; de Esch et al., 2012; Helmcke et al., 2010; Leung et al., 2008; McGrath and Li, 2008; Nishimura et al., 2015; Peterson et al., 2008; Roper and Tanguay, 2018; Sedensky and Morgan, 2018)).

The zebrafish has been widely used in developmental, genetic and neuroscience research. The zebrafish genome is highly similar to other vertebrates with 71.4% of human genes having zebrafish orthologs and sharing a 60–80% homology (Howe et al., 2013; Renier et al., 2007). In addition, some regions of the zebrafish nervous system show structural homology to the relevant areas of the human brain, including the hypothalamus, optic tracts, olfactory system, spinal cord and cranial nerves (Mueller and Wullimann, 2003). Moreover, zebrafish have an endothelial tight junction-based blood–brain barrier that was shown to be similar to that of higher vertebrates (Jeong et al., 2008). Additionally, zebrafish and mammals share a similar set of neurotransmitters, such as GABA, glutamate, dopamine, noradrenaline, serotonin, histamine and acetylcholine (Panula et al., 2010). Taken together, the nervous system of zebrafish shows similarities to mammalian counterparts at the anatomical, physiological and molecular level, rendering the zebrafish as a suitable model for assessing DNT. With regard to metabolic capacity, it is important to note that interspecies differences exist and not all members of the mammalian drug-metabolizing CYP families and subfamilies have been identified in zebrafish. However, it has been shown that zebrafish express CYP3A enzymes, which are homologous to the human CYP3A subfamily (McGrath and Li, 2008), one of the most common CYP isoforms for human metabolism. Zebrafish larvae can grow in multiwell plates due to their small size and can rapidly develop major organs and tissues by 5 days post fertilization, making them suitable for high-throughput screening. Embryos and larvae of zebrafish are transparent until 7 days post fertilization allowing for visualization of developmental processes (Richards et al., 2008). More importantly, studies of zebrafish neural and behavioral functions have led to the establishment of models that assess sensory, motor and cognitive functions as well as models for CNS deficits, such as anxiety, depression, addiction, autism and obsessive-compulsive states (see reviews (de Esch et al., 2012; Meshalkina et al., 2017)). This highlights the possibility of combining biochemical, molecular and cellular assessments with observations of behavioral functions in zebrafish for DNT testing.

C. elegans has also been widely studied in genetics and nervous system development. The genome of *C. elegans* comprises approximately 20,000 genes with homologues for 60–80% of human genes (Kaletta and Hengartner, 2006). *C. elegans* has a very well-characterized nervous system with 302 neurons, 1410 neuromuscular junctions and 6393 neurotransmitter driven synapses (Avila et al., 2012). The synapses are of the same types as those in mammals, including cholinergic, GABAergic, glutamatergic, dopaminergic and serotonergic. In addition, the *C. elegans* model provides several advantages for high-throughput neurotoxicity screening, such as small size (1.3 mm), ease of culture in the laboratory, short generation time (3 days), large brood size (over 300 progeny per self-fertilizing hermaphrodite), transparency allowing for visualization as well as the ease of making reporter gene fusions. During the past decade, increasing studies have used *C. elegans* as a model to assess neurotoxicity of compounds at molecular, structural and behavioral levels. A wide variety of compounds assessed in these studies include selenium, isoflurane, acrylamide, nicotine, metals (such as manganese, cadmium, MeHg, iron, and arsenic) and pesticides (such as paraquat, rotenone and organophosphates) (see reviews (Avila et al., 2012; Sedensky and Morgan, 2018)). Results from these studies to some extent emulated the findings in mammalian systems, demonstrating that the *C. elegans* model could provide useful insights into the neurotoxicity potential of chemicals.

Taken together, despite some inter-species differences in molecular, cellular and functional characteristics, there are some advantages to using an intact organism for DNT testing. In comparison with *in vitro* models, the organism-based models include a metabolic component, represent a physiologically relevant microenvironment for neurodevelopment, take into account toxicokinetics, and are able to assess behavioral and cognitive functions. For zebrafish and *C. elegans* in particular, both species have short life cycles and are inexpensive to maintain, therefore suitable for high-throughput screening. More importantly, the nervous system of these species has a high degree of evolutionary conservation with mammals, enabling the generation of reliable and relevant DNT insights. Indeed, DNT assessments in zebrafish based on neuron apoptosis, teratogenicity, and embryonic/larval locomotor activity demonstrated a high concordance with mammalian data by using training sets of compounds (Selderslaghs et al., 2013; Ton et al., 2006). Further verification of concordance with mammalian DNT

findings in *C. elegans* and zebrafish models using large sets of chemicals will further establish the applicability of these models for DNT testing.

3. A proposal for a practical testing strategy

3.1. Introduction of the AOP concept

In light of the growing need for more efficient methods to assess hazards, the field of toxicology testing is undergoing a paradigm shift from primarily relying on apical observations in animals toward incorporating alternative methods with the existing knowledge of biological pathways that are responsible for adverse outcomes, namely, adverse outcome pathways (AOP). The concept of AOP is to construct a framework that describes the mechanistic linkage of molecular, cellular, organ and organism responses triggered by a toxicant exposure. As such, a typical AOP begins with a molecular initiating event (MIE), which depicts the initial interaction of a chemical with its biological target. The MIE triggers a sequential series of key events (KE) that represent the molecular, cellular, structural and/or functional changes in biological processes, which ultimately result in a specific adverse outcome (AO) in individuals and populations (Ankley et al., 2010). The knowledge-based AOP framework provides novel opportunities for the application of alternative testing methods in chemical hazard identification under the premise that a pathway responsible for a KE *in vivo* should also be similarly responsive in an *in vitro* assay reflecting the same KE (Bal-Price et al., 2015a). Additionally, the mechanism-focused nature of AOP provides the knowledge base for the selection of the most effective tests to cover MIEs and KEs, and therefore guiding the assembly of integrated testing strategies to meet regulatory needs.

3.2. Overview of DNT-related AOPs

The development of AOPs for DNT is a newly emerging but fast-growing field. In order to provide a comprehensive overview of currently available AOPs pertaining to DNT, available AOPs were collected here (Table 2). Those AOPs include the ones that were specifically developed for DNT (*i.e.*, AOP 2, 3, 5, 10, 11, 12, 13, 14, 15 and 16 in Table 2) and the ones that were originally developed for neurotoxicity but have potential to be applied at the developmental stage (*i.e.*, AOP 1, 4, 6, 7, 8, 9, 17 and 18 in Table 2). For those neurotoxicity AOPs that are not specific for DNT, the MIE and/or some KEs could occur during the neurodevelopment stage and hence could be potentially relevant to DNT. Therefore, those AOPs were also included from the standpoint of establishing an inclusive knowledgebase that covers, as broad as possible, potential DNT mechanisms. The detailed information regarding these AOPs can be found in previous review papers (Bal-Price et al., 2015b, 2017; Sachana et al., 2018; Terron et al., 2018), US EPA report (EPA, 2017) as well as AOP Wiki (https://aopwiki.org/aops, accessible at Dec 21, 2018), a component of the OECD-sponsored AOP Knowledgebase effort. These AOPs covers a wide range of MIEs, including the interactions of chemicals with neuron-specific receptors and enzymes (*e.g.*, AOP 1, 2, 3, 4, 17 and 18), binding to SH-/seleno- proteins (AOP 5), interruptions of the electron transport chain in the mitochondrion (*e.g.*, AOP 6, 7, 8 and 9), and interferences with thyroid hormone biosynthesis, metabolism and transportation (*e.g.*, 10, 11, 12, 13, 14, 15 and 16). Given the large number and the diversity of the MIEs, it is challenging and even unpractical for a single laboratory to designate a set of testing methods for each of the 18 AOPs in order to determine the DNT potential of chemicals in development. Therefore, a practical solution may be focusing on common key events (CKE) (Bal-Price et al., 2015a) that represent the converging key events triggered by multiple MIEs and thus serving as surrogates for the activation of multiple AOPs. By choosing those CKEs as testing endpoints, the number of assays can be reduced to a manageable number, which makes the assembly of an integrated testing strategy more feasible and practical for a single lab. Toward this end, we aligned a graphic representation of all 18 AOPs to identify CKEs (Fig. 1). The AOPs are grouped into clusters based on the similarity in MIEs and/or KEs. As shown in the figure, the flow of KEs initiated from divergent MIEs start to converge at cellular and organ effect levels as they proceed toward an adverse outcome. Specifically, the majority of KEs from AOPs 1–7 lead to mitochondrial dysfunction and subsequent cell injury/death, which in turn leads to neurodegeneration and altered neuronal network function. Likewise, increased NAD^+ in AOPs 8 and 9 results in changes in neural stem/progenitor cell (NS/PC) differentiation and ultimately leads to altered neuronal network function as well. In addition, AOPs 10–16 depict the perturbations of biosynthesis, metabolism and transportation of thyroid hormone, which then lead to decreased thyroxine (T4) in the brain and ultimately alter neurodevelopmental processes, including neuronal proliferation, apoptosis, migration, neurite outgrowth and neuronal network connectivity (Zoeller and Rovet, 2004). Lastly, AOPs 17 and 18 describe the modulations of sodium channel and GABA receptor activity, which result in altered activity in neuronal networks. Taken together, the integrated graphic representation of 18 AOPs (Fig. 1) reveals 5 CKEs (highlighted in orange) that can potentially serve as initial testing endpoints for DNT evaluation. Those CKEs include: (I) mitochondrial dysfunction; (II) cell injury/death; (III) altered neuronal network function/activity; (IV) altered differentiation of NS/PCs into neurons and glia cells; and (V) decreased T4 in neuronal tissue. As additional DNT-related AOPs are developed, these pathways can be integrated into the strategy to identify other CKEs and improve the breadth of DNT screening.

3.3. Proposal for an integrated testing strategy for DNT testing

Identifying CKEs represents the first step toward developing an efficient screening program for a single laboratory with an integrated testing strategy framework for DNT evaluation. The following step is to select a set of assays that can be used to assess those CKEs (Table 3). As discussed in previous section of currently available alternative models, cell viability (CKE I and II) is one of the well-established and commonly measured endpoints in a variety of *in vitro* models that quantifies apoptotic and necrotic cell death as well as mitochondrial dysfunction including changes in ATP content and reactive oxygen species. Although cell injury/death is not a unique toxicological endpoint for DNT, it is recognized that neurons and glia may be inherently more sensitive to neurotoxic agents during the development stage. In addition, activation of neuron-specific receptors/pathways by chemicals that do not cause cytotoxicity in other cell types may result in neuronal cell death (*e.g.*, excitotoxicity). Therefore, combining assessments of specificity (*e.g.*, QSAR, cheminformatics, read across) and relative sensitivity (*e.g.*, comparative dosimetry) to aid in the interpretation of injury/death in developing neurons and glia would provide useful information to infer DNT potential of chemicals. In addition, the differentiation of NS/PC into neurons and glia cells (CKE IV) can be assessed by measuring cell lineage-specific markers in both 2D and 3D cell based models. Moreover, the neuronal network activity (CKE III) can be measured in hESC-/iPSC-derived neural culture using MEA technique. One limitation of cell-based *in vitro* models is that they cannot be used to evaluate tissue thyroxine level (CKE V), as they lack cellular/tissue components required for thyroid hormone biosynthesis, metabolism and transportation. As a complement to this deficit, a non-mammalian organism-based model, such as zebrafish, can be used to measure alterations of thyroxine level (CKE V) after chemical exposure (Zhai et al., 2014). In addition, organism-based models can provide further insights into the behavioral changes resulting from DNT.

Based on the discussion above, here we propose an integrated testing strategy for DNT testing that can be used by individual laboratories. Because AOPs contain the explicit information of MIEs (for instance, the binding of agonists to glutamate receptor NMDAR (AOP 4)), it is possible to utilize *in silico* modeling to evaluate the potential of chemicals to trigger given MIEs based on the structural and chemico-

Table 2
Currently available DNT and neurotoxicity AOPs.

AOP #	Specific for DNT?	Title	Molecular initiating event	Adverse outcome	Reference
1	No	Binding of inhibitors to acetylcholinesterase trigger neuroinflammation leading to neurodegeneration	Binding of inhibitors to acetylcholinesterase	Neurodegenetive disorders	Bal-Price et al., 2015b
2	Yes	The interaction of non-dioxin-like polychlorinated biphenyls with ryanodine receptors causes their sensitization affecting neuronal connectivity that results in behavioral deficits	Binding of non-dioxin-like polychlorinated biphenyls with ryanodine receptor	Behavioral deficits (learning and memory, psychomotor attention)	Bal-Price et al., 2015b, 2017
3	Yes	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	Binding of antagonists to N-methyl-D-aspartate receptor during synaptogenesis	Impairment of learning and memory	Bal-Price et al., 2015b, 2017; Sachana et al., 2018; AOPWIKI[a] (AOP13)
4	No	Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment	Binding of agonists to ionotropic glutamate receptor	Impairment of learning and memory	Bal-Price et al., 2015b; AOPWIKI[a] (AOP48)
5	Yes	Binding of electrophilic chemicals to the SH(thiol)-group of proteins and/or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	Binding to SH-/seleno-proteins involved in protection against oxidative stress	Impairment of learning and memory	Bal-Price et al., 2015b; AOPWIKI[a] (AOP17[b])
6	No	Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits	Binding of inhibitor to NADH-ubiquinone oxidoreductase (complex I)	Parkinsonian motor deficits	Bal-Price et al., 2015b; Terron et al., 2018; AOPWIKI[a] (AOP3)
7	No	Interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration	Interaction uncouplers with oxidative phosphorylation	Neurodegenetive disorders	Bal-Price et al., 2015b
8	No	Interaction of redox cycling chemicals with NADH-quinone oxidoreductase results in NAD+ formation causing reduced adult neurogenesis	Binding of redox cycling chemicals NADH-quinone oxidoreductase	Learning and memory deficits	Bal-Price et al., 2015b
9	No	Interaction of redox cycling chemicals with NADH cytochrome b5 reductase results in NAD+ formation causing reduced adult neurogenesis	Binding of redox cycling chemicals with NADH cytochrome b5 reductase	Learning and memory deficits	Bal-Price et al., 2015b
10	Yes	Sodium iodide symporter (NIS) Inhibition and subsequent adverse neurodevelopmental outcomes in mammals/Inhibition of NIS leads to learning and memory impairment	Sodium iodide symport inhibition	Cognitive function deficits/learning and memory impairment	AOPWIKI[a] (AOP134[b]/AOP54[b]) US EPA report (2017)
11	Yes	Inhibition of thyroperoxidase and subsequent adverse neurodevelopmental outcomes in mammals	Thyroperoxidase inhibition	Cognitive function deficits	AOPWIKI[a] (AOP42) US EPA report (2017)
12	Yes	Upregulation of thyroid hormone catabolism *via* activation of hepatic nuclear receptors, and subsequent adverse neurodevelopmental outcomes in mammals	Xenobiotic nuclear receptor activation	Neurological dysfunction, hearing loss	AOPWIKI[a] (AOP8[b]) US EPA report (2017)
13	Yes	Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	Interference with thyroid serum binding protein	Cognitive function deficits	AOPWIKI[a] (AOP152[b]) US EPA report (2017)
14	Yes	Inhibition of deiodinase results in decreased thyroxine (T4) to triiodothyronine (T3) conversion and subsequent adverse neurodevelopmental outcomes	Deiodinase inhibition	Neurological and cognitive impairments	US EPA report (2017)
15	Yes	Interference with thyroid receptor and subsequent adverse neurodevelopmental outcomes	Thyroid receptor binding	Neurological and cognitive impairments	US EPA report (2017)
16	Yes	Interference with thyroid hormone transport results in decreased T4 in brain and subsequent adverse neurodevelopmental outcomes	Thyroid hormone transport interference	Neurological and cognitive impairments	US EPA report (2017)
17	No	Binding of pyrethroids to voltage-gated sodium channels induces acute neurotoxicity	Binding of pyrethroids to voltage-gated sodium channels	Type I syndrome: Hyper-excitability, tremor and hyper-reactivity. Type II syndrome: choreoathetosis, salivation	Bal-Price et al., 2015b, 2017
18	No	Binding of antagonist to γ-aminobutyric acid receptor $GABA_AR$ results in hyperexcitability and convulsions[c]	Binding of antagonist to $GABA_AR$	Seizures/convulsions	Bal-Price et al., 2015b

[a] AOPWIKI is available at: https://aopwiki.org/aops (accessible at December 12, 2018).
[b] AOP under development. In cases where the AOPs in AOP wiki that are under development and not ready to cite, the information regarding MIE, KE, AO and graphic representation of those AOPs are taken from alternative references other than AOP wiki.
[c] GABAR is relevant to neurodevelopment but the response varies with developmental stages (Bal-Price et al., 2015b).

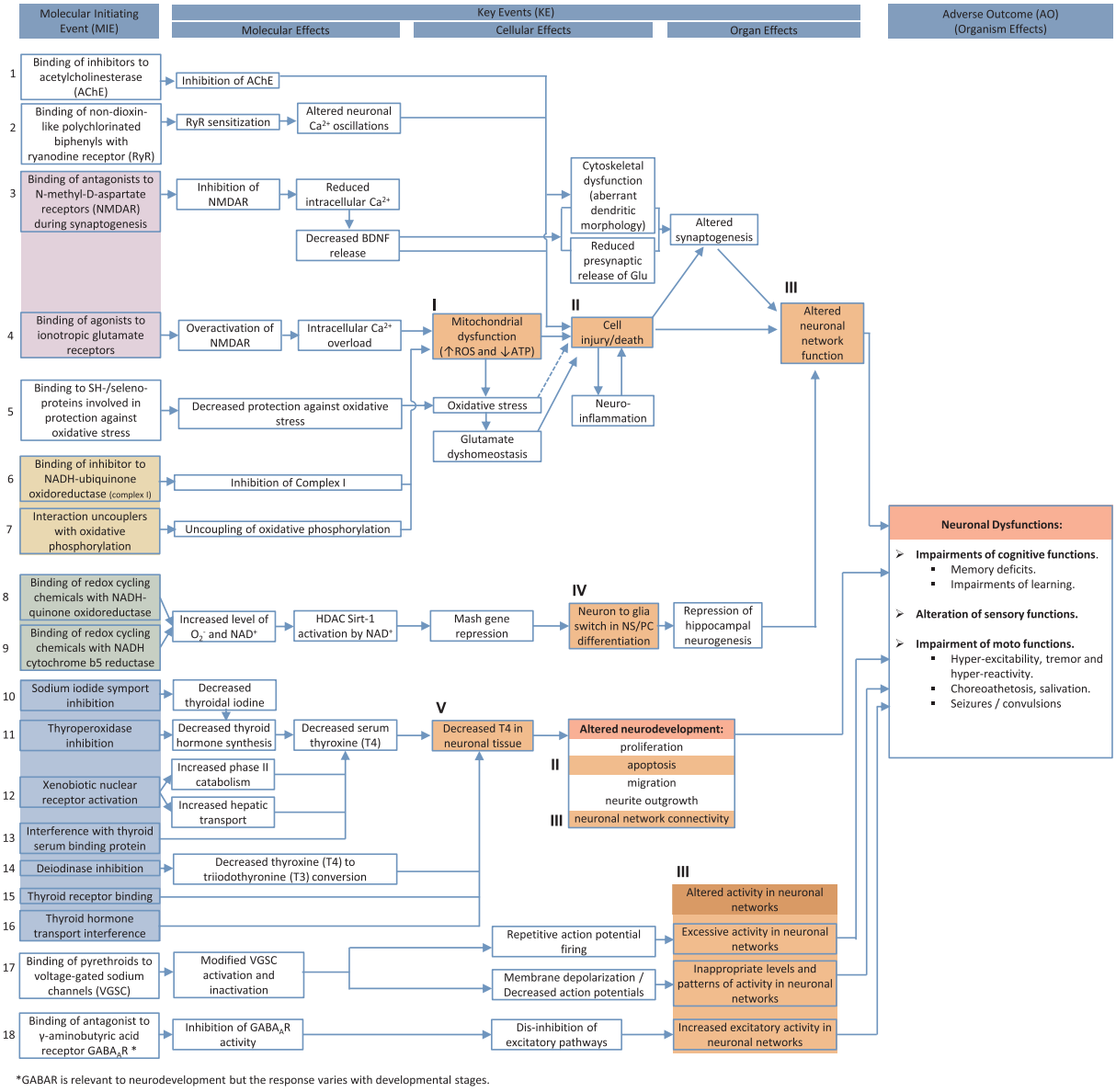

Fig. 1. Integrated graphic representation of 18 DNT-related AOPs. All of 18 currently available DNT-related AOPs are aligned and grouped into color-coded clusters based on the similarity in molecular initiating events (MIEs) and/or key events (KEs). Identified common key events (CKEs) are numbered and colored in orange. This graphic representation is created for the purpose of trying to identify commonalities between AOPs. Although it covers the majority of KEs, this graphic representation does not purport to be complete and is likely to change as AOPs are revised and new AOPs are developed. The detailed information for each AOP can be found in references listed in Table 2. As additional DNT-related AOPs are developed, this figure can be updated to identify additional CKEs. BDNF, brain-derived neurotrophic factor; ROS, reactive oxygen species; NAD, nicotinamide adenine dinucleotide; HDAC, histone deacetylase; NS/PC, neural stem/progenitor cell.

physical properties. Some of the examples include the cheminformatic models currently under development in-house that predict the binding of chemicals to acetylcholinesterase, acetylcholine or GABA receptors, *etc.* based on chemical structure. This *in silico* approach can be used to prioritize chemicals to be tested and select more targeted *in vitro* assays for follow-up testing. In addition, it is critical to incorporate exposure data and toxicokinetics in this integrated testing strategy for the purpose of risk assessment (Blaauboer, 2003). Toxicokinetics integrates information of absorption, distribution, metabolism and excretion, therefore can be used to determine *in vitro* testing concentration ranges that are relevant to realistic exposure scenarios. This approach will help to reduce false-positive findings due to overloading the *in vitro* systems with chemical concentrations that far exceed realistic exposure levels. Moreover, toxicokinetics provides information on possible metabolites that might be tested instead of the parent compound and therefore minimize false-positives and false-negatives due to lack of maternal, placental and fetal metabolism in the *in vitro* systems (Fritsche, 2017). Taken together, toxicokinetics modeling (*e.g.*, *In vitro* to *In vivo* Extrapolation (IVIVE) modeling or physiologically based pharmacokinetic (PBPK) modeling) are needed to determine contextual *in vitro* testing concentration ranges and potential metabolites and better facilitate subsequent *in vitro* testing. The following testing comprises a battery of human cell-based *in vitro* assays that assess the effects of chemicals on mitochondrial function (CKE I), neural cell injury/death (CKE II), neuronal network function (CKE III) and neural differentiation (CKE IV). If disturbance of CKEs is detected, further testing can be streamlined toward these effects in non-mammalian organism-based models (*e.g.*, zebrafish). Meanwhile, the change in thyroxine level (CKE V) can also be assessed in zebrafish. The aforementioned alternative methods (*in silico*, *in vitro* and non-mammalian organism-based models) represent more time- and resource-efficient approaches when compared to traditional animal-based testing, and can be used to identify DNT

Table 3
Alternative model assays that can be used to assess common key events identified in DNT-related AOPs.

AOP #	Common key events	Testing approaches							
		2D Cell-based models			3D Cell-based models			Organism-based models	
		Cell viability assay	Neural differentiation assay	Neuronal network activity assay (MEA)	Cell viability assay	Neural differentiation assay	Neuronal network activity assay (MEA)	Zebrafish	*C. elegans*
1									
2	− Mitochondrial dysfunction	√			√			√	√
3									
4	− Cell injury/death	√			√			√	√
5									
6	− Altered neuronal network function			√			?	√	√
7									
8	− Altered NS/PC differentiation		√			√		√	√
9									
10									
11	− Decreased thyroxine (T4) in neuronal tissue							√	
12									
13	− Cell injury/death	√			√			√	√
14									
15	− Altered neuronal network function			√			?	√	√
16									
17	− Altered activity in neuronal networks			√			?	√	√
18									

AOP # refers to AOPs listed in Table 2. √ indicates the capability of the alternative model assay to assess the corresponding common key event. ? indicates the capability of the alternative model assay is under development.

alerts and prioritize chemicals for further testing when screening chemicals during development. In cases where *in vitro* or non-mammalian organism-based models indicate a concern for potential DNT or those models are deemed to be unsuitable based on certain criteria (such as low sensitivity/specificity, difficult to test substance due to insolubility or irrelevant route of exposure), an *in vivo* rat study may be used as a higher tiered test for DNT assessment.

Taken together, the proposed integrated testing strategy consists of three tiers of testing. The *first tier* is *in silico* which includes cheminformatic models and toxicokinetics models. The *in silico* approaches will provide information regarding the potential MIE of chemicals and help to prioritize and streamline chemicals for better targeted follow-up *in vitro* testing. Positive and negative prediction models will provide greater assurance when prioritizing or deprioritizing compounds for additional screening/testing. IVIVE modeling also helps to determine relevant *in vitro* testing concentrations. The *second tier* consists of human-cell based *in vitro* models to assess the DNT potential of prioritized chemicals by evaluating the changes in CKEs. It is noteworthy to mention that data generated by *in vitro* testing can be used to feedback to and refine *in silico* models including a determination of effect levels relative to exposures. Finally, the *third tier* involves organism-based models (including zebrafish and rat), which can serve as confirmatory, complementary or more definitive testing for *in vitro* assays. Likewise, the data generated in the third tier testing can be used to evaluate the performance of *in vitro* and *in silico* assays. These iterative processes between models in different tiers will be a valuable asset for improving the integrated testing strategy.

3.4. Limitations and path forward

There are some limitations for the proposed integrated testing strategy for DNT testing. First, the proposed strategy is developed based on the currently available AOPs, which could not possibly capture all of molecular mechanisms underlying DNT. In addition, there are some shortcomings of the AOP concept, including that some MIEs and KEs often comprise a large number of biochemical events (*e.g.*, oxidative stress, inflammation), lack effect duration or a time window component, deal with multiple hit events and event modifiers, and inadequately consider compensatory mechanisms (Leist et al., 2017). These limitations generally make screening with identified AOP KEs and CKEs more conservative for detecting potential chemical interactions. Secondly, *in silico* models for many of the 18 MIEs have not been developed yet due to lack of definitive understanding of molecular mechanism and the lack of sufficient high-quality data. Thirdly, the applications of current and to-be-developed *in vitro* assays are yet to be fully recognized, including reproducibility, sensitivity, specificity, accuracy and domain of applicability. As with any *in vitro* system, separating direct activity from non-specific cytotoxic and/or cell stress-related alterations remains a challenge.

It is noteworthy to mention that the proposed strategy is developed based on the current state of knowledgebase of DNT. Although this knowledgebase does not necessarily capture all of the DNT mechanisms, the current effort represents the first step of finding commonalities between AOPs and using identified CKEs to build a practical testing strategy. In future, as the understanding of DNT advances, more and more AOPs will be developed and can then be incorporated into this approach to extend its applicability. In addition, increasing data sets obtained from *in vitro* bioactivity testing are become available, which can be used to develop *in silico* models that explore the correlations between chemical structure and the potential of triggering MIEs. Moreover, there are a growing number of collaborative efforts worldwide to validate and assess the performance of *in vitro* DNT models.

Better characterization of the assay performance will strengthen the confidence in the *in vitro* testing and may someday make it possible to conduct risk assessment solely based on *in silico* and human *in vitro* assays. Ultimately, the proposed integrated testing strategy as a whole must be challenged with a large set of well-characterized training chemicals to evaluate its performance and identify gaps for further improvement.

4. Conclusion

By reviewing the current knowledgebase of DNT AOPs, a set of CKEs are identified which may present efficient endpoints for DNT screening by allowing for maximum coverage of modes of action with minimum assay development and validation. Based on those CKEs, a tiered approach is proposed consisting of targeted *in vitro*/alternative model assays as well as cheminformatic, exposure and IVIVE modeling. This integrated strategy, when validated using training chemicals, can be a practical DNT screening approach that is efficient to detect a wide variety of potential modes of action when examining commercial/industrial chemicals with unknown DNT potential.

Transparency document

The Transparency document associated with this article can be found in the online version.

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