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In vitro acute and developmental neurotoxicity screening: an overview of cellular platforms and high-throughput technical possibilities

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Abstract Neurotoxicity and developmental neurotoxicity are important issues of chemical hazard assessment. Since the interpretation of animal data and their extrapolation to man is challenging, and the amount of substances with information gaps exceeds present animal testing capacities, there is a big demand for in vitro tests to provide initial information and to prioritize for further evaluation. During the last decade, many in vitro tests emerged. These are based on animal cells, human tumour cell lines, primary cells, immortalized cell lines, embryonic stem cells, or induced pluripotent stem cells. They differ in their read-outs and range from simple viability assays to complex functional endpoints such as neural crest cell migration. Monitoring of toxicological effects on differentiation often requires multiomics approaches, while the acute disturbance of neuronal functions may be analysed by assessing electrophysiological features. Extrapolation from in vitro data to humans requires a deep understanding of the test system biology, of the endpoints used, and of the applicability domains of the tests. Moreover, it is important that these be combined in the right way to assess toxicity. Therefore, knowledge on the advantages and disadvantages of all cellular platforms, endpoints, and analytical methods is essential when establishing in vitro test systems for different aspects of neurotoxicity. The elements of a test, and their evaluation, are discussed here in the context of comprehensive prediction of potential hazardous effects of a compound. We summarize the main cellular characteristics underlying neurotoxicity, present an overview of cellular platforms and read-out combinations assessing distinct parts of acute and developmental neurotoxicology, and highlight especially the use of stem cell-based test systems to close gaps in the available battery of tests.

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APC	Automated patch-clamp
ASD	Autism spectrum disorders
BBB	Blood–brain barrier
CMP	Cell membrane potential
CNS	Central nervous system
DA	Dopaminergic
DNT	Developmental neurotoxicity
ECs	Endothelial cells
ENT	Engineered neural tissue
ER	Endoplasmic reticulum
EST	Embryonic stem cell test
FI	Fluorescence intensity
FTD	Frontotemporal dementia
hESCs	Human embryonic stem cells
hiPSCs	Human-induced pluripotent stem cells
HT	High throughput
IATA	Integrated approaches to testing and assessment
ITS	Integrated testing strategies
KE	Key event
MEA	Multielectrode array or microelectrode array
NPC	Neural progenitor cell
NT	Neurotoxicity
OECD	Organisation for Economic Co-operation and Development
PD	Parkinson's disease
PNS	Peripheral nervous system
SOD1	Cu/Zn-binding superoxide dismutase
TALEN	Transcription activator-like effector nucleases

Chemical hazard assessment is changing

Regulatory toxicology is undergoing a major transition from an observational discipline based mostly on animal experiments to a mechanism-based science embracing also in vitro experimentation (Hartung and Leist 2008; Hartung 2009).

This change involves a movement from the current 'black box' approach of animal experiments towards a new approach of risk assessment using mainly in vitro testing to identify the mechanism of toxicity ('adverse outcome pathways') (Leist et al. 2008b; Hartung and McBride 2011; Bhattacharya et al. 2011; Leist et al. 2014).

Many practical considerations support this evolution. First, concordance between animal and human toxicity can be surprisingly low: rodents have been found to be predictive of human toxicity in less than half of the cases (Olson et al. 2000) and teratogenicity in humans seems to be especially poorly predicted by animal studies (Nau 1986; Basketter et al. 2012). Second, in vitro safety testing allows for the compounds be tested at more realistic concentrations than the high concentrations commonly used in animal testing in order to minimize the number of animals needed. Third, reducing the need for animals and implementing high-throughput methods should both lower the cost and increase the reliability of safety testing.

If we consider neurotoxicology more specifically, there are also scientific reasons that support complementing or replacing the current methods employing animals with in vitro testing using human cells. Although the human brain is not as different from other animal brains in its basic composition as once thought (Azevedo et al. 2009), it is not just a 'large rodent brain' (Herculano-Houzel 2011). For instance, there are some species-specific differences in the microarchitecture of the neocortex, and the human one seems to have a higher proportion of inhibitory (GABAergic) neurons and more asymmetrical synapses per neuron than in rats or mice (DeFelipe et al. 2002).

Mouse and human brains also differ in the temporal aspects of neurogenesis (Florio and Huttner 2014), which is important for developmental neurotoxicity (DNT). Although the majority of developmentally important genes is evolutionary conserved, their spatial and temporal expression patterns significantly differ between rodent and human developing brains (Fougerousse et al. 2000).

Certain cell types found in the human brain have not been identified in rodent brains (Allman et al. 2011). For example, Von Economo neurons (not present in the brain of rodents) are selectively lost in the brains of people with frontotemporal dementia (FTD) (Seeley et al. 2006) and in individuals with a history of alcoholism (Senatorov et al. 2015).

Astrocytes play a major role in response to the toxicants. In humans, they are more abundant (Nedergaard et al. 2003; Efremova et al. 2015), larger, have 10 times more processes and different signalling (Zhang et al. 2016), and are organized in more complex domains than their rodent counterparts. Varicose projection and interlaminar astrocytes are present in human but not in rodent brains (Oberheim et al. 2009).

There are also some differences between the functioning of microglia in humans and mice that are relevant to neurotoxicology: murine microglia respond more readily to inflammatory stimuli with nitric oxide production, as the iNOS promoter shows species differences (Chu et al. 1998).

The most important differences between human and rodent brains arise from the different primary sequence of

human proteins and their rodent orthologs. This affects not only receptor affinities and enzyme recognition, but also the inflammatory response (Leist and Hartung 2013). The Toll-like receptors (TLRs) that play important functions in innate immunity provide a good example: the expression pattern of TLRs is different in mice and man, and they respond differently to lipopolysaccharides (Mestas and Hughes 2004). Also, due to the presence of some non-conserved histidine residues in the human sequence, TLR4 triggers for instance contact hypersensitivity to nickel in humans, but not in mice (Schmidt et al. 2010). Differences in the primary sequences between orthologs can also lead to differences in the selectivity of the transport proteins of the blood–brain barrier leading to differences in the uptake of certain compounds (Liu et al. 2015).

There may also be significant differences between murine and human disease biology, as a systematic comparative study of gene expression changes in burns, trauma, and endotoxemia suggested (Seok et al. 2013). The authors found unexpectedly poor correlation between gene expression changes in about 5000 human genes and their mouse orthologs (although the extent of difference seems to depend on the statistical method) (Takao and Miyakawa 2015; Warren et al. 2015). The differences in murine disease models and human disease become most apparent from the vast clinical failure of drugs that work in animal models (Leist and Hartung 2013).

Besides the above-mentioned practical and scientific considerations, there is also legislative pressure to implement alternative in vitro safety testing methods for cosmetics products (European Parliament 2009). The European chemicals legislation REACH (Hartung 2010; Rudén and Hansson 2010) also provides a strong impetus for developing in vitro platforms for toxicity testing, by the sheer number of animals that would be necessary for complying with it (Höfer et al. 2004; Hartung and Rovida 2009). While neurotoxicity is not a typical stand-alone endpoint in these legislations, it represents a key organ manifestation in systemic toxicity testing, and there are specific regulatory needs especially for plant protection products (Coecke et al. 2006).

The three primary guidelines of the Organisation for Economic Co-operation and Development (OECD) covering neurotoxicity and DNT are OECD 424 (neurotoxicity study in rodents), OECD 426 (developmental neurotoxicity study), and OECD 443 (extended one-generation reproductive toxicity study). The functional tests and clinical observations in these guidelines are similar to those specified in OECD Guidelines 407 (rodent 28-day repeated dose oral toxicity studies) and 408 (rodent 90-day repeated dose oral toxicity studies), but employ a larger sample size and call for more frequent evaluation of functional tests.

The purpose of the OECD guidelines is to identify chemicals that permanently or reversibly affect the nervous system, to characterize any chemical-induced alterations in the nervous system, and to estimate dose levels for regulatory uses. Specific endpoints to evaluate functional, behavioural, and morphological effects of the nervous system in all study types include (1) detailed clinical observations in the home cage and open field; (2) neurofunctional tests including motor activity; and (3) neuropathology using perfusion-fixed tissues. Additional testing specifically for offspring that have been exposed in utero and during early lactation includes sensory function testing, sexual maturation (OECD 426 and OECD 443), assessments of behavioural ontogeny, and learning and memory (OECD 426). However, these tests are not run by default for all chemicals. On the contrary, most DNT information is missing even for high-production-volume chemicals (Bal-Price et al. 2015a). Neurotoxicity studies are not usually run for REACH chemicals, unless there is a clear indication from other studies that there is a hazard for the nervous system. DNT studies are mandatory for pesticides in Europe; in the USA, they are only required when there is evidence of neurotoxicity. Thus, there is a large need especially for screening assays that can be used as initial indication of neurotoxicity or DNT, and that may be used for further testing prioritization.

Significant funding has been allocated for reforming toxicology both in the USA and in EU countries; for example, the recent Horizon 2020 EU-ToxRisk project aims at developing non-animal-based methods for toxicity testing, and the US Tox21c and ToxCast projects have similar objectives (Tice et al. 2013; Kleinstreuer et al. 2014).

Features facilitating neurotoxicity and developmental neurotoxicity

There are six specific properties which affect special toxicity in the nervous system compared with other organs: (1) separation of the CNS from the blood stream by the blood–brain barrier (BBB), (2) the special lipid-rich composition of the brain and the nerves, (3) the high energy requirement of the central nervous system (CNS), (4) the specific intercellular signal transmission, (5) the special morphology and structure of neural cells, and (6) the specific biochemistry of neurons. An additional issue is that ‘neurotoxicity’ usually does not manifest throughout the nervous system, as may be observed for hepatotoxicity in the entire liver, or bone marrow toxicity throughout the bone marrow structures. In the case of neurotoxicity (NT), a very specific and small area affected can lead to a dramatic loss of function; for example, loss of substantia nigra dopaminergic neurons

(<0.001 % of all neurons) leads to Parkinson's disease, and poisoning of a very small population of interneurons in the spinal cord can lead to lethality by tetanus toxin.

The blood–brain barrier

It protects the brain against various compounds, including neurotoxicants (Cecchelli et al. 2007; Alépée et al. 2014). However, it is not impermeable for all substances, since there is directed transport, diffusion of lipophilic substances over cell membranes, and paracellular passage of water-soluble substances across tight junctions (Hawkins and Davis 2005; Abbott et al. 2010; Zhao et al. 2015). Some central neurotoxicity may arise from precursor substances (such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) that cross the BBB and are then metabolically activated within the CNS, by astrocytes (Schildknecht et al. 2015). Notably, as there is no BBB in the circumventricular organs and in the peripheral nervous system, not all neurons in the body are protected by the BBB (Miyata 2015). This explains in part the selective peripheral neurotoxicity of compounds such as doxorubicine. Therefore, when performing and interpreting neurotoxicity studies, the ability of a substance to cross the BBB should always be evaluated (Alépée et al. 2014). Furthermore, the blood–brain barrier of the developing nervous system is less efficient, which makes the immature brain more susceptible to certain chemicals (Kadereit et al. 2012; Zimmer et al. 2012; Balmer et al. 2012; Krug et al. 2013b; Waldmann et al. 2014).

Lipid-rich structures

The high ratio of membrane to cytoplasm of neurons, and the additional amount of membranes coming from the myelin sheets formed by Schwann cells or oligodendrocytes also increase the vulnerability of the nervous system to special toxicants. Membranes are mainly built from lipids, and they therefore accumulate lipophilic substances, e.g. methylmercury (Lohren et al. 2015).

Energy requirement

Neurons require a high amount of ATP for generating and maintaining their membrane potential. Additionally, due to their long processes, they need more energy for directed transport of organelles, such as mitochondria (Zsurka and Kunz 2015; Gibbs et al. 2015) and of components of the translational machinery (Fernandez-Moya et al. 2014). The dependence on oxidative phosphorylation as the major source of energy makes neurons highly vulnerable to inhibitors of the mitochondrial respiratory chain, such as rotenone (Ayala et al. 2007) and carbon monoxide (Bunc et al. 2006).

Synaptic transmission

Signal transmission from one neuron to another is the key function of the nervous system. The release of neurotransmitters into the synaptic cleft and the binding of neurotransmitters to the receptors on the postsynaptic membranes are required to transmit the signals. Successful packaging of presynaptic vesicles and the clearance of neurotransmitters from the synaptic cleft are critical for functional signal transmission. Therefore, all these processes are potential targets for neurotoxicants: neurotransmitter release may be inhibited (e.g. by botulinum neurotoxins, which block the fusion of vesicles with the membrane), receptors may be affected (e.g. curare and nicotine affect the nicotinic acetylcholine receptor) or the clearance of neurotransmitters can be impaired (e.g. organophosphates block acetylcholine esterase activity) (Berliocchi et al. 2005; Marrs and Maynard 2013). Effects on signal transmission in the adult nervous system will give rise to acute toxicity; altered signal transmission during the nervous system development may permanently affect structure and function of the nervous system.

Neural cell structure

Compared with other cell types, neurons have long projections called axons. Axons of peripheral neurons can reach a length that exceeds the diameter of the cell body by a factor of >100,000. Such distances pose special demands on intracellular transport (Gibbs et al. 2015). Substances, such as taxol, that disturb cytoskeletal elements and impair this transport cause peripheral neuropathy (Quasthoff and Hartung 2002; Hoelting et al. 2016).

Neurobiochemistry

A property making some subgroups of neurons more sensitive to toxicants than neighbouring cells is their specific biochemistry. For example, the dopamine neurotransmitter synthesized in dopaminergic neurons autoxidizes quickly. Substances that increase the release of dopamine (e.g. methamphetamine) or inhibit its reuptake or breakdown (e.g. cocaine) increase the autoxidation rate of dopamine and the resulting radicals damage the cells (Lotharius et al. 2005; Schildknecht et al. 2009). Another factor explaining the neuro-specific adverse effects of trimethyltin is the expression of the protein stannin (Toggas et al. 1992).

Cell maturity also determines the susceptibility to neurotoxicants (Leist and Nicotera 1998; Alépée et al. 2014). This is the crossing point of neurotoxicology and developmental neurotoxicology. The development of the nervous system requires several tightly controlled processes (e.g. neural tube formation and closure) that are regulated by

sensitive signalling pathways. These are very susceptible to even small disturbances (Penschuck et al. 2006; Kuegler et al. 2010; Zimmer et al. 2011a; Kadereit et al. 2012; van Thriel et al. 2012).

All these points should be considered when selecting the cellular platforms, read-outs, and the methods to test for acute or chronic NT and for DNT. The neuronal subtype-specific toxicity of some chemicals suggests that one platform, one read-out, and one method are not enough to effectively predict DNT and NT.

Understanding the elements of in vitro neurotoxicity test systems

As basis for the methods, endpoints, and cell models described here, it appears important to provide a framework of how they fit into a toxicological test set-up. For this purpose, it is essential to recall the basic four elements that always make up any toxicological test: the biological system, the endpoint, the exposure scheme, and the prediction model (Fig. 1). Separate chapters are devoted to the first two elements (Chapters 4 and 5), while the exposure scheme and prediction model are discussed below (Chapters 3d and 3e, respectively,) and in Chapter 7.

The elements of a toxicological test are often confused, as the terms to describe these elements are used inexactly in the literature. The definitions as used, e.g. by the European validation authority (EURL-ECVAM), are outlined and exemplified below.

Test (or test method)

This term is used in many disciplines, and it is little defined in colloquial language. In toxicology, it means a procedure based on a test system used to obtain information on the potentially hazardous effects of a substance. A toxicological test method consists of four major components (i.e. test system, exposure scheme, endpoint, prediction model), and it produces a test result (information regarding the ability of a substance or agent to produce a specified biological/toxicological effect under specified conditions). The terms ‘test’ and ‘assay’ are used interchangeably in the literature. A test method can have several analytical endpoints.

Test system/biological system

This term is often confused with ‘test method’, but it has a different definition. A test system is a cellular (or biochemical) system used in a study (e.g. ‘proliferating neural

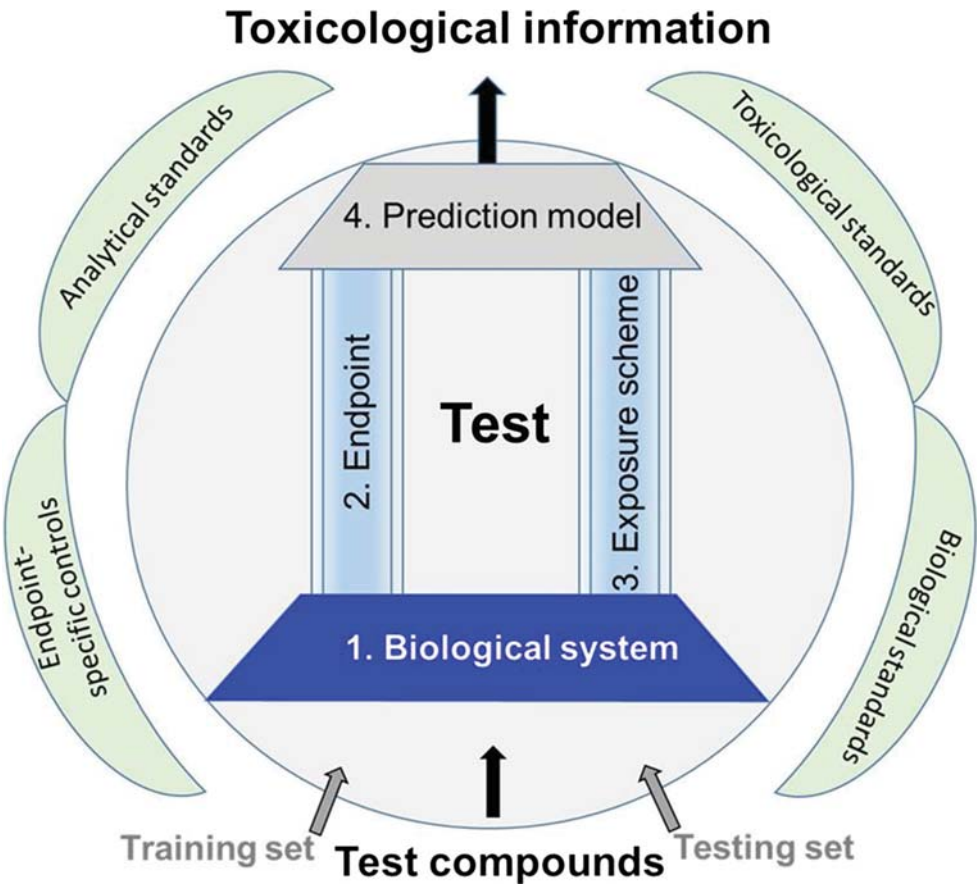

Fig. 1 Elements of a test system, and the frame to calibrate it

stem cells', or 'neuronally differentiating PC-12 cells', or 'organotypic hippocampal slices'). The term is often used interchangeably with 'in vitro system'. The test system is equivalent to 'biological model' as far as test set-up is concerned [see (1) in Fig. 1]. Thus, the test system is only one component of a test or test method. Good performance of a test system does not imply good functioning of a test method. Acceptability criteria for test systems (e.g. at least 75 % of the differentiated cells staining positive for nestin under control conditions) are different from acceptability criteria for the test method using the test system (e.g. inhibition of differentiation by a specified positive control by at least 35 %, and alteration of normal differentiation by a defined negative control by <10 %).

Test endpoint/analytical endpoint

The term endpoint has two implications, and it is essential to understand the differences. Within the context of a toxicological test, the endpoint (or 'test endpoint') is the biological or chemical process, response, or effect assessed in a test system by a specific analytical method/assay [see (2) in Fig. 1]. For instance, 'cell viability' or 'cell proliferation' or 'electrical network activity' are endpoints. Each endpoint may be assessed by different analytical methods, i.e. using different 'analytical endpoints'. For instance, 'viability' may be assessed by measurement of LDH release, resazurin reduction, by cell counting, or by measurement of ATP content. 'Differentiation' may be measured by PCR quantification of a differentiation marker or by morphometry (e.g. dendritic tree arborizations or synaptic spine density). Chapter 5 contains more examples of common endpoints and the different analytical methods that can be used to measure them. It is important to distinguish analytical endpoints (referring to the analytical methods used) from (test system) endpoints that refer to the biological concept evaluated. The test endpoint and analytical endpoints require independent optimization, characterization, and use of control compounds (see also Chapters 3f–3i for more detail).

The above definitions cover the first two elements of a toxicological test (dealt with in this review). The other two elements are discussed here to plan better for the incorporation of test systems and endpoints in new toxicological tests.

Exposure scheme

A drug may be added to a test system continuously, or for certain time periods, in a certain solvent, with or without medium change, at a specified temperature, etc. All this information is contained in the exposure scheme [see (3) in Fig. 1]. As each of the other three elements of a test, an exposure scheme also needs to be optimized independently. For instance, with all other test parameters fixed, the test outcome can dramatically change with the time period of exposure. Depending on the point of view, the analytical endpoint may be regarded as part of the exposure scheme. Optimization of the exposure scheme may require switching analytical endpoints, even if the same test endpoint is evaluated.

Prediction model

The prediction model (PM) is a formula or algorithm (e.g. formula, rule, or set of rules) used to convert the results generated by a test method into a prediction of the (toxic) effect of interest [see (4) in Fig. 1]. Sometimes, the PM is also referred to as decision criteria.

A prediction model contains four elements: (1) a definition of the specific purpose(s) for which the test method is to be used; (2) specifications of all possible results that may be obtained; (3) an algorithm that converts each study result into a prediction of the (toxic) effect of interest; and (4) specifications as to the accuracy of the prediction model (e.g. sensitivity, specificity, and false–positive, and false-negative rates).

In this context, the 'data interpretation procedure (DIP)' is of interest also. It signifies any algorithm for interpreting data from one or more information sources. The output of a DIP is typically a prediction (e.g. prediction of skin sensitization potential from peptide binding data and/or chemical structure).

The PM is often neglected in test set-up. In its narrow sense, it defines the procedure how data are being processed, and how technical data (instrument readings) are translated into toxicological information. For instance, if calcium oscillations are measured, the PM determines what type of change is considered as relevant to toxicity. Another important example is a change in gene expression, measured by PCR or a transcriptomics approach. A heatmap of gene expression is a technical set of data, but not toxicological information. A PM transforms this into a test statement of compound hazard. A first consideration about PM is whether there is a binary outcome (toxic–non-toxic) or are there more than 2 classes (mild, moderate, severe irritants, and how are the boundaries defined). For instance, many in vitro tests give information if a compound is hazardous or non-hazardous, but not on the strength of effect or the potency of a chemical.

Another important issue is if there are two or more assay endpoints (e.g. viability and neurite growth), how are they combined to a final toxicity statement? During test optimization and validation, the prediction model needs scrutiny, and the questions asked are as follows: Is there a threshold (different from the statistical threshold) for when an effect can be considered biologically relevant? How is the outcome interpreted when more than one endpoint is measured (e.g. general cytotoxicity and functional impairment

or effects on two different cell types)? Is an increase compared to normal good, when a decrease is bad? How should data be interpreted when a compound alters the baseline values for the endpoint (e.g. coloured compound in spectrophotometric assays)? What is the correct reference value, if the test system changes over time? The PM defines these decision points and then translates the test result into a prediction, e.g. converting the luminometer reading of an ATP assay into a toxicological statement (prediction) whether the compound is cytotoxic (at a given concentration).

In practical terms, a test is set up to be predictive for unknown compounds (test compounds), but to achieve this goal, the different elements of the test usually require optimization and fine-tuning. This is performed by anchoring the test or its elements to a frame of known information, i.e. defined controls and standards as outlined below.

Analytical standards

Each analytical method requires calibration by the use of standards (positive and negative controls). This can include physicochemical approaches (e.g. to make sure that the balances and the spectrophotometer are working), or scaling approaches (e.g. to obtain absolute values in microscopic morphometric measurements or counts). On the next level, the analytical endpoint needs to be calibrated in the context of the test system. For instance, if LDH release is used as a measure of viability, then it needs to be evaluated, how much LDH is released under conditions of all cells dying (e.g. detergent lysis; not necessarily = 100 %), and the overall assay needs to be normalized to such values. An important example is viability measurement by resazurin or tetrazolium dye reduction. This works only after normalization for cells that are 100 % dead or alive, as the instrument readings as such have no dimension.

Endpoint-specific controls

These are chemicals known to reliably and consistently alter the endpoint of a test system at a mechanistic level. They are also referred to as 'endpoint-selective controls' or 'mechanistic tool compounds'. This would be the first set of compounds, used during test system set-up, to obtain information on the biological/toxicological behaviour of the test system and its dynamic range. Such control compounds can be used to define acceptance criteria.

Positive/negative control (PC/NC) or 'toxicological standards'

A PC is a compound or condition that triggers a response, i.e. a change in the endpoint from baseline in the right direction and to a certain specified extent. A NC for a 'test method' is a compound or condition that should not trigger a response, i.e. it should not change the endpoint from baseline. The performance of PC and NC can be used to define 'acceptance criteria' of a test.

Acceptance criteria

Criteria are defined before performing an assay to determine whether it is 'valid', i.e. whether the data can be used. Typical issues of acceptance criteria comprise: 'has the actual run or plate of the test method functioned (e.g. are the endpoint values for PC and NC in the right range)', 'is the test method performing within the desired range of variability (e.g. are the standard deviations of PC and NC in the right range)'. Note that acceptance criteria can (and should) also be defined for an 'analytical endpoint' or for a 'test system'. For instance, for a test system, the acceptance criteria may say that it is only valid if at least 400 cells were in the region of interest, or if at least 80 % neurons were present in mixed cultures, or if the average neurite length was at least 4 cell diameters. Such test system acceptance criteria are not at all related to those used for the test method. In this context, it is important to rationalize that endpoints that are meaningful for the description of the test system/biological system may not be useful for the test method and vice versa. For instance, a person's body weight can be measured well on scales (to give a good read-out on general growth characteristics of a person = biological system), but this endpoint will hardly respond to acute poisoning of the person. Instead, blood pressure or vomiting activity may be good measures of human poisoning (toxicological test), but they in turn give little information on the growth activity over time. In a neurotoxicity test for network activity, the extent of synaptic staining may be a good acceptability criterion for the test system, but it will not react to a glutamate receptor agonist; on the other hand, electrical activity pattern will be a very sensitive measure for glutamate receptor-affecting toxicants, but the synapse number will not change (upon acute exposure)

Once the first three elements of the test system have been established, optimized, and assembled to a test, the prediction model can be established to complete the test system set-up. One standard procedure is to use a training set of chemicals. Based on the test data, a prediction model would be established that suits best the known information about which of the compounds should test positive or negative. In a second round of testing, a test set of compounds would be used (i.e. a new set of positive and negative controls). The data of these substances would be run through the prediction model to determine accuracy, specificity, and sensitivity of the test system. Possibly, further adaptations would then follow.

Cellular test systems used for neurotoxicity testing

Available cellular platforms range from non-human primary cells to human-induced pluripotent stem cell (hiPSC)-derived neurons, and include complex co-cultures and 3D systems (see Table 1 for a summary).

Non-human primary neural cells, neural cell lines, and embryonic stem cells

For neurocytotoxicity screens, primary cultures of mixed cell types (e.g. re-aggregated spheroids (Honegger et al. 1979; Monnet-Tschudi et al. 2007) or enriched cultures of neurons (Volbracht et al. 1999; Gerhardt et al. 2001; Suñol et al. 2008; Valdivia et al. 2014), or astrocytes (Ni et al. 2012) are frequently used.

To circumvent the necessity to prepare new cells from animals for each new experiment, immortalized neuronal-like cell lines of murine and rat origin have been generated (Greene and Tischler 1976; Davis and Maher 1994; Alwin Prem Anand et al. 2012). One example of a cell model for HTS screening is the multipotent C17.2 murine neural progenitor cell line (Snyder et al. 1992). This cell line has the ability to differentiate into a mixed cell culture consisting of neurons and astrocytes

Table 1 Cell sources and test systems

Model system	Strength(s)	Weakness(es)
Non-human		
Primary cells (dissociated brain cells of rodent embryos/pups)	Functional neuronal circuits in 2 weeks or less	Heterogeneous cell population, new animals needed for each culture, labour intensive
Rodent neuronal cell lines (e.g. C17.2 murine immortalized neural progenitor cell line, Neuro-2a murine neuroblastoma cell line, PC12, IMA)	No animals needed, less labour	Immortalized cells or cells originated from tumours—physiology not human, and it may be altered, limited relevance
Mouse embryonic stem cells	Self-renewing, pluripotent, validated platform for DNT	Non-human physiology, limited relevance
Human		
Primary neurons	Maybe a gold standard	Limited availability
Primary astrocytes	Commercially available	Variable quality and characterization
Immortalized cell lines (e.g. LUHMES)	Easily expandable, standardized differentiation protocols to neurons, astrocytes, and oligodendrocytes exist	Physiology may be altered by oncogene/telomerase overexpression
Cell lines isolated from neoplasms (e.g. SH-SY5Y human neuroblastoma cell line)	Easily expandable, can be induced to more differentiated phenotype	Physiology may be altered; abundant genetic aberrations
Neural stem cells	Shown to survive, migrate, and differentiate in vivo, commercially available, well characterized, robust	Ethical concerns, culture expensive, unclear which developmental state of brain region they represent, differentiation protocols may be lengthy
Embryonic stem cells (hESC)	Self-renewing, pluripotent, can form complex structures, differentiation protocols to several cell types have been established, becoming a widely accepted alternative to animal testing for DNT	Ethical concerns, culture expensive, differentiation protocols may be lengthy
Induced pluripotent stem cells (hiPSC)	Self-renewing, pluripotent, can form complex structures, can model different human genetic backgrounds, differentiation protocols to several cell types have been established	Culture expensive, differentiation protocols may be lengthy May show large differences from line to line due to human genetic background variability and clonal selection
BBB models	Combination of relevant cell types, e.g. microvascular endothelial cells, astrocytes, and pericytes Measurement of toxicokinetic parameters Assessment of damage to the BBB function	Need to establish organize 3D structure Need for sophisticated analytical endpoints (no high throughput)
Organs-on-a-dish (3D structures)	Interaction of several cell types 3D contact points Opportunity to observe myelination and neuroinflammation Opportunity to observe tissue self-organization as function	Complex analysis (no high throughput) Difficult quality control

(Lundqvist et al. 2013), and may also serve as a model for developmental neurotoxicity studies. The major sources of cell lines are either neoplasms (neuroblastomas, phaeochromocytomas, e.g. Neuro-2a; PC-12, HT22) (Tischler and Greene 1975; Dypbukt et al. 1992; Repetto et al. 1994; Behl et al. 1995), or cells immortalized by oncogenes (e.g. IMA) (Schildknecht et al. 2012; Efremova et al. 2015).

Embryonic stem cells (ESCs) are pluripotent cells capable of continuing self-renewal and with the ability to differentiate into any cell type in the body. They were first isolated from the inner cell mass of blastocyst stage embryos of mice (Evans and Kaufman 1981; Martin 1981), later also from human origin pre-implantation blastocysts (Thomson et al. 1998; Leist et al. 2008a).

The potential of using murine embryonic stem cells (mESCs) for in vitro developmental toxicity testing was recognized long time ago (Heuer et al. 1993), and the murine ESC-based embryonic stem cell test (EST) became a validated platform for embryotoxicology testing (Genschow et al. 2004; Hayess et al. 2013). Robust protocols have been developed for neural differentiation of mESC and are in use for DNT (Kuegler et al. 2010; Zimmer et al. 2011a, b; Theunissen et al. 2012; Visan et al. 2012; Smirnova et al. 2014a, c; Colaianna et al. 2016).

Human primary cells and immortalized human neural progenitor cell lines

In order to avoid species extrapolation in toxicity testing, human primary neural cells have also been used (Hansson et al. 2000). Access to such human cells is limited, since they are derived from aborted foetuses or from brain surgery resections. Moreover, large variability is found amongst cell batches. Nevertheless, protocols to derive highly pure populations of neurons, microglia, oligodendrocytes, and astrocytes from foetal human brains have been developed (Jana et al. 2007).

A way of circumventing the mentioned limitations has been to generate immortalized cell lines from neurons obtained from the mesencephalon (Lotharius et al. 2002; Paul et al. 2007; Selenica et al. 2007; Miljan et al. 2009; Villa et al. 2009; Scholz et al. 2011), the cortex (Donato et al. 2007), the subventricular zone (Bai et al. 2004), or from the spinal cord (Roy et al. 2007). This can be achieved either by overexpression of the myc oncogene, or—to avoid the use of oncogenes—overexpression of human telomerase. These neuronal progenitor cell lines provide an expandable cell base that can generate large numbers of cells. New opportunities arise from cultivating such cells in 3D organoids (Smirnova et al. 2015a).

Several human cell lines have also been isolated from tumour tissue. The SH-SY5Y human neuroblastoma cell line is one of the most frequently used neuronal cell models in screening assays. The cells can be used in their native form, or be differentiated to more pronounced neuronal phenotypes (Påhlman et al. 1984).

Human neural stem cells present an alternative source. These cells can be expanded in vitro, differentiated to various cell types, and used for (developmental) neurotoxicity testing (Gassmann et al. 2012; Barenys et al. 2016).

Human embryonic stem cells (hESCs)

As we mentioned above, the murine embryonic stem cell test has become a validated test for DNT (Seiler and Spielmann 2011). The species-difference issue led to the development of humanized in vitro toxicology screens using hESCs (Pellizzer et al. 2005; Adler et al. 2008b). For example, Stummann et al. demonstrated that methylmercury [a known embryotoxicant that was initially difficult to classify by the EST (Genschow et al. 2004; Theunissen et al. 2010)] had a strong effect on neuronal precursor formation on hESCs subjected to a neuronal differentiation protocol (Stummann et al. 2009). Pal et al. used a few model drugs to explore what endpoint measurements would be most useful for predicting DNT with hESCs, settling on apoptosis markers, abnormal expression of germ-layer-specific differentiation markers, and hormone levels (Pal et al. 2011).

Several studies have explored the use of hESC derivatives for developmental toxicology testing (Adler et al. 2008a; Krtolica et al. 2009; West et al. 2010; Zimmer et al. 2014; Dreser et al. 2015; Pallocca et al. 2016). Balmer et al. used hESCs differentiating to neural cells to explore the mechanism of action of valproic acid (VPA), an antiepileptic drug with well-documented DNT effects (Balmer et al. 2012). This system allowed them to use concentrations close to the VPA concentration expected in the foetus in vivo (Waldmann et al. 2014). The studies have been extended to comprise a large group of HDAC inhibitors, and the results demonstrated that the system is suitable for toxicant classification and for epigenetic studies (Balmer et al. 2012, 2014; Balmer and Leist 2014; Shinde et al. 2015; Rempel et al. 2015).

hESCs have been differentiated into many defined neuronal subpopulations (e.g. dopaminergic, glutamatergic); such dopaminergic neurons were sensitive to a known neurotoxicant causing Parkinson's disease-like symptoms in humans (Zeng et al. 2006).

A functional test based on hESC-derived neurons monitored neurite outgrowth by automated high-content screening and proved the feasibility of such an approach to chemical safety assessment (Harrill et al. 2010; Hoelting et al. 2015).

Human-induced pluripotent stem cells (hiPSC) and induced neurons (iNeurons)

The research group of Yamanaka was the first to achieve the reprogramming of murine somatic cells into pluripotent cells in 2006 (Takahashi and Yamanaka 2006). A year later, reprogramming of human somatic cells to pluripotent cells, known as human-induced pluripotent stem cells (hiPSCs) was also accomplished (Takahashi et al. 2007; Yu et al. 2007). Today, the reprogramming of somatic cells, including human cells, using defined factors and diverse methodologies is well established (Malik and Rao 2013; Raab et al. 2014).

There is a large body of evidence showing that hiPSCs share most characteristics of hESCs (Hu et al. 2010; Nicholas et al. 2013). Their ability for self-renewal and their pluripotency make hiPSCs an unlimited source of practically all cell types of the body. The envisaged uses range from cell replacement therapy through drug-screening and disease modelling to toxicity testing (Phillips and Crook 2010; Wobus and Löser 2011; Kolaja 2014)

The most important challenge in using hiPSC derivatives for toxicity screening purposes is to produce cells that model closely cells found in vivo. A large number of protocols for the generation of different neuronal cell types has been published (for an overview, see Fig. 2). Not always can established hESC neuronal differentiation protocols be applied to differentiate hiPSCs, because all pluripotent cells (hESC and iPSC) differ amongst one another (Hu et al. 2010). The most likely explanation for this is the variation in genetic background (Müller et al. 2011; De Los Angeles et al. 2015; Tsankov et al. 2015). Thus, published hESC neural differentiation protocols usually need to be adapted for a specific iPSC line.

As early as 2007, the group of S. Yamanaka differentiated hiPSCs into neuronal cells, including dopaminergic neurons (Takahashi et al. 2007). In 2009, the group of L. Studer introduced a feeder-free protocol for differentiating midbrain dopaminergic and spinal motor neurons based on SMAD inhibition (Chambers et al. 2009). Additionally, hiPSC-specific differentiation protocols for cholinergic neurons (Karumbayaram et al. 2009), forebrain-type interneurons (Nicholas et al. 2013), and cortical glutamatergic neurons (Vazin et al. 2014), but also for astrocytes, and oligodendrocytes (Yan et al. 2013; Nguyen et al. 2014; Gorris et al. 2015) have been established.

Instead of the chemical environment, genetic manipulation may be used for efficient differentiation. Forced expression of a single transcription factor (such as Neurogenin-2 or ASCL1) is sufficient to convert hiPSCs into synaptic network-forming neuronal cells [see Fig. 2; (Zhang et al. 2013; Chanda et al. 2014)].

A recent development is the direct conversion of somatic cells into neurons (iNeurons) without passing through the pluripotent stage (Pfisterer et al. 2011; Chanda et al. 2013; Wapinski et al. 2013). This approach might provide faster, simpler, and cost-effective generation of neurons; however, the full characterization and validation of the resulting neuronal properties will be crucial for practical applications. In this very dynamic field, it can be expected that many refined, specific protocols will be established in the near future.

It is essential for a cell platform to be used for in vitro neurotoxicity screening that the cells are available in large amounts and in consistent quality. This need is being addressed by a growing number of companies. ReproCELL was the first to provide a human hiPSC-derived neural progenitor cell (NPC) kit, consisting of NPCs and the necessary reagents to differentiate these NPCs into functional dopaminergic neurons. Cellular Dynamics International, Axogenesis, Thermo Fisher, and other companies and university laboratories offer human hiPSC-derived neural cells. Many cell types derived from iPSC are now available commercially. For example, Cellular Dynamics International (Madison, USA) has developed iCell Neurons, a highly pure population of human neurons derived from hiPSCs comprising a mixed population of GABAergic and glutamatergic neurons. Because iCell Neurons exhibit key cellular and electrophysiological characteristics of neurons with high reproducibility, they seem well suited for electrophysiological recordings and have been used on Nanion's automatic patch-clamp (APC) device, the Patchliner. Na^+, K^+, and Cl^- conductances were characterized by APC in neurons, and at an acceptable success rate compared to manual patch-clamp (Haythornthwaite et al. 2012).

Models of the blood–brain barrier (BBB)

The term blood–brain barrier (BBB) indicates the specialized endothelial lining of brain microvasculature that controls the transit of water-soluble compounds between the peripheral circulation and the brain parenchyma. Recently, the BBB has also been called the 'neurovascular unit' because of the tight association of cells from the parenchymal side of the basement membrane (astrocytes, neurons, pericytes) with the endothelial cells (Bauer et al. 2014). It is important to know for the development of in vitro models that soluble factors provided by astrocytes determine BBB function, morphology, and protein expression pattern.

Attempts to fabricate the solid support for BBB models (Prieto et al. 2004) include the use of transwell inserts (Wang et al. 2015; Hind et al. 2015), hollow fibres made of thermoplastic polymers (Cucullo et al. 2011b), 3D printing (Kim et al. 2015), and photolithography (Arayanarakool

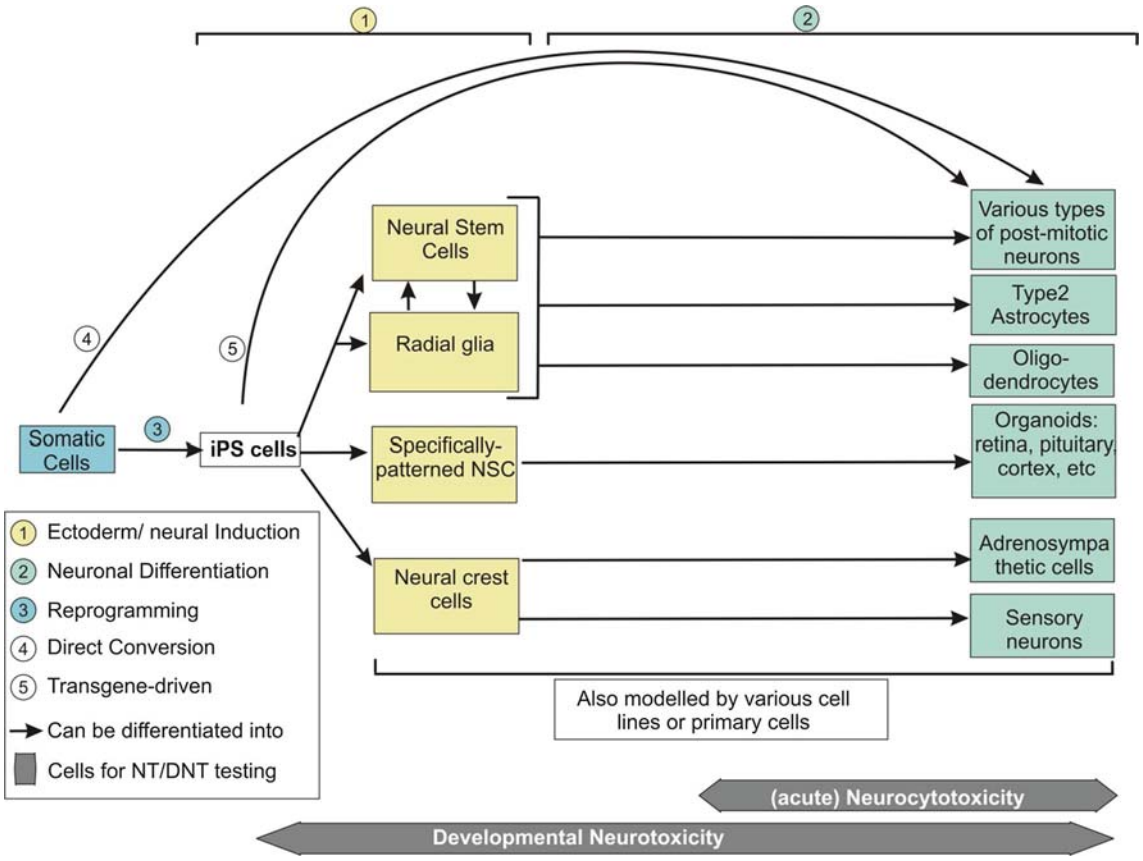

Fig. 2 Overview of neuronal differentiation from hiPSCs and possible in vitro models

et al. 2015). Creating in vitro models that mimic all BBB functions is particularly difficult, as endothelial cells (ECs) require exposure to shear stress to undergo the differentiation required for a well-functioning BBB (Cucullo et al. 2011a).

In vitro models of the BBB are highly desirable since they would reduce the cost of drug permeability testing and provide a simpler and more controllable working environment (Naik and Cucullo 2012). Culot et al. found that their in vitro BBB model can be used as toxicological screen in a high-throughput setting (Culot et al. 2008). One model of the BBB—consisting of human ECs grown on the luminal side of capillaries bearing microholes, co-cultured with human astrocytes on the abluminal surface—was able to reproduce several key features of the BBB including high trans-endothelial electrical resistance, low permeability to sucrose, capability of discriminating between solutes with different lipophilicity, an increased propensity for aerobic metabolism, and even opening in response to hyperosmotic mannitol treatment or allowing for trans-endothelial trafficking of monocytes from the luminal to the parenchymal compartment of the model (Cucullo et al. 2011b).

Since differences between human and rodent isoforms of the transport proteins expressed by ECs mean that certain compounds can be substrates for the human isoform, but not for the rodent counterpart, it is important to use cells of human origin for BBB models to get results that translate to human safety (Liu et al. 2015).

Organ-in-a-dish models

There has been tremendous progress in developing 3D organoid models of the brain (Alépée et al. 2014; Andersen et al. 2014). Current models of brain tissue include re-aggregated differentiated rodent brain cells, neurospheres generated from neural stem cells or neural progenitors, and engineered neural tissue (ENT) differentiated from hESCs or hiPSCs (Monnet-Tschudi et al. 2007; Preynat-Seauve et al. 2009; Dubois-Dauphin et al. 2010; Hogberg et al. 2013; Lancaster et al. 2013; Tieng et al. 2014; Simão et al. 2015; Paşca et al. 2015; Materne et al. 2015; Terrasso et al. 2015; Mariani et al. 2015; Simão et al. 2016).

Using a microfluidic system for 3D cultures of human neuroepithelial stem cell-derived dopaminergic (DA)

neurons is a new technical approach (Moreno et al. 2015). DA neurons were differentiated directly within the inlet wells of the system. The system holds potential not only to investigate cytotoxicity, but also DNT aspects. Many other neuronal cells types can be tested if protocols for their differentiation can be adjusted to the system.

Bioprinting is an alternative method to spontaneously forming neuronal tissue is the generation of 3D tissues (Lee et al. 2009; Murphy and Atala 2014). Two different strategies have been utilized for neurotoxicity testing: (1) a 3D scaffold consisting of biocompatible materials was printed, and cells were seeded on it (the tissue formed by cells migrating into their position); (2) cells were printed together with a biocompatible substrate. Either pluripotent cells (ESCs and iPSCs) or multipotent neural stem cells (NSCs) can be used for printing. Subsequently, neuronal maturation takes place within the scaffold and can be influenced by specific growth factors (Lee et al. 2010). In the future, this method can provide extremely well-defined 3D constructs for neurotoxicity as well as DNT assays. Additionally, the interaction of specific cell types printed onto the same scaffold can be monitored to answer more complex questions of cytotoxicity.

Combining brain tissue models with BBB models has been attempted in order to set up a general in vitro testing platform for neurotoxicity. Shultz et al. generated their BBB model using bovine endothelial cells co-cultured with rat glial cells that separated the 'luminal compartment' from murine neuronal networks (a 2D model) or rat re-aggregating brain cell cultures (a 3D model) on the 'parenchymal' side in the well (Schultz et al. 2015). This integrated testing system allowed analysis of whether the drug passed or damaged the BBB, as well as metabolomics, transcriptomics, proteomics, and functional (e.g. multielectrode array; MEA) read-outs of the neurons.

Common endpoints of neurotoxicity testing

Hundreds of targets may be affected by neurotoxicants. Since it is not practical to develop one assay for every molecular initiating event (MIE), downstream key events (KE) corresponding to essential neuronal functions affected by several MIEs provide a more economic basis for in vitro neurotoxicity testing (Bal-Price et al. 2008; Galofré et al. 2010; Gustafsson et al. 2010; de Groot et al. 2013; Bal-Price et al. 2015a, b).

The endpoints used for in vitro neurotoxicity testing can be grouped into viability read-outs, morphological read-outs, and functional read-outs (see Fig. 3 for examples). Viability read-outs can identify neurotoxic compounds that cause toxicity in one or more neural cell types at lower concentration than they affect viability of other cell types. Perturbations caused at sub-cytotoxic concentrations can be identified by morphological or functional read-outs.

The endpoints used for in vitro neurotoxicity screens include assessment of cell viability, evaluation of neuronal differentiation (e.g. measuring neurite outgrowth and biomarker expression), monitoring spontaneous electrical activity of neuronal networks by MEA, and functional read-outs like receptor signalling and cell communication, migration, gliosis, network formation, and synaptogenesis. A summary of common endpoints is found in Table 2. The distinction between morphological and functional read-outs can be blurry and depends largely on the analytical method used for measuring the endpoint—in these cases, endpoints are listed under functional endpoints in Table 2.

Cell viability

Energy metabolism can be monitored by measurement of ATP content of cells (Volbracht et al. 1999; Latta et al. 2000), or quantifying the reduction in resazurin or tetrazolium salts (e.g. MTT) by viable cells (Krug et al. 2014; Pamies et al. 2014; Schildknecht et al. 2015). The release of lactate dehydrogenase (LDH) from dead cells (Leist et al. 1996; Wong et al. 2001) is also amenable for high-throughput use and an indicator of irreversible cell damage.

The disadvantage of these assays is that in cultures consisting of several cell types, cell death of one type of cells can be masked by proliferation of another type of cells. For example, the resazurin assay failed to detect the death of neurons in primary cultures of rat cerebellar granule cells exposed to methyl mercury chloride because of the glial proliferation triggered by the neuronal cell death (Hogberg et al. 2010). For this reason, methods that involve direct counting of cells are in widespread use. They can be used in combination with immunofluorescent labelling or morphometric methods to identify particular cell types. Some viability measures require lysing of the cells, so the cells cannot be used for further assays. The LDH release assay and the resazurin assay maybe performed non-invasively. The resazurin reduction assay can also be multiplexed with other assays, such as measurement of the activities of caspase-3 and caspase-7.

Measuring neuroapoptosis (programmed neuronal cell death) is a special case of monitoring changes in cell viability due to chemical exposure (Leist and Jäättelä 2001). The apoptosis process involves complex biochemical events carried out by a family of cysteine proteases called caspases that can be activated by various stimuli (Thornberry and Lazebnik 1998; Orrenius et al. 2003). The hallmarks of apoptosis include mitochondrial damage (Cory and Adams 2002), plasma membrane alterations (externalization of

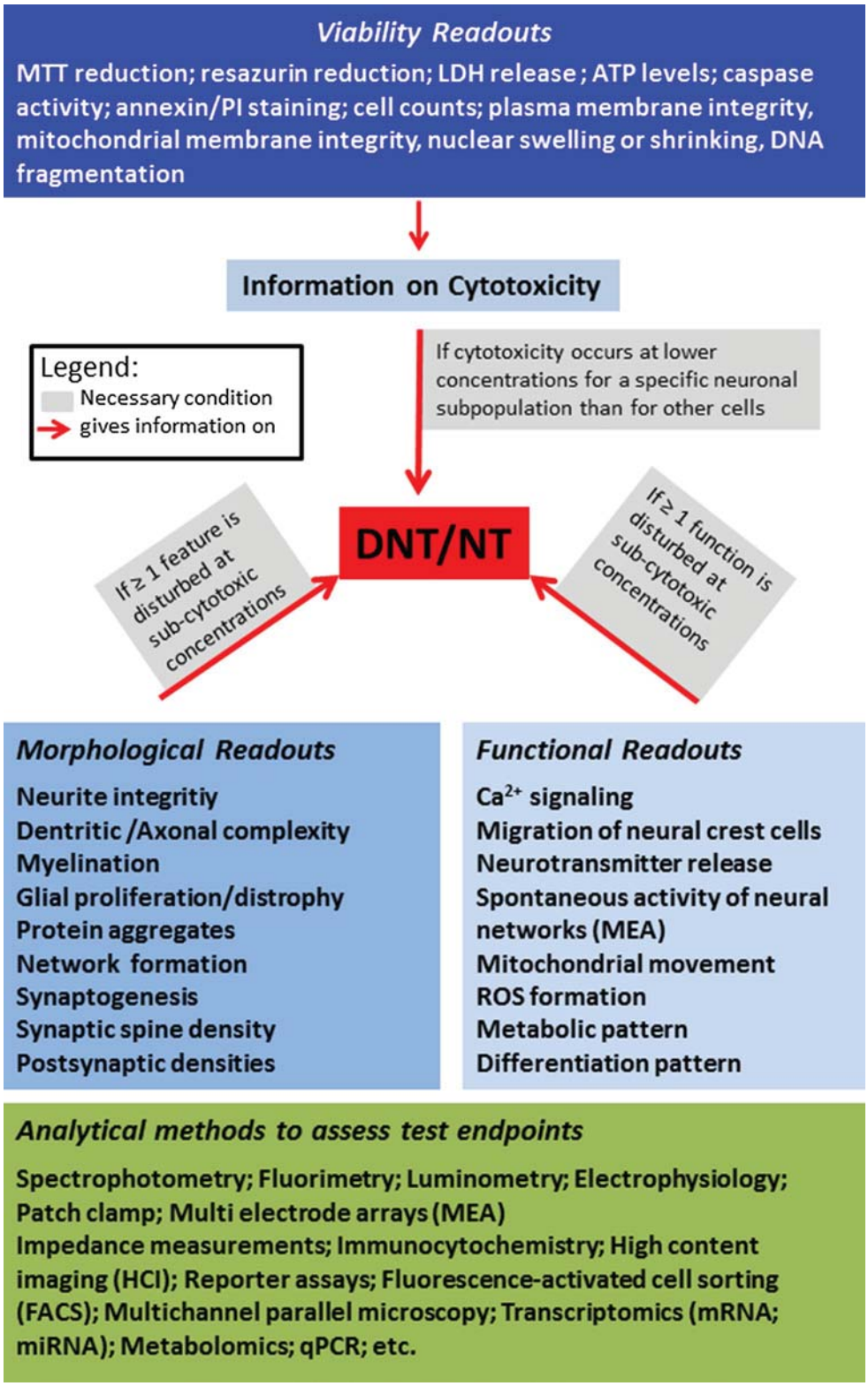

Fig. 3 Experimental approaches to define specific (developmental) neurotoxicity

phosphatidylserine) (Leist and Jäättelä 2001), and DNA fragmentation. Environmental toxicants can cause developmental neurotoxicity by either stimulating or inhibiting apoptosis (Rice and Barone 2000).

For HT detection of apoptosis, fluorescence and light microscopy can be used (Steinbach et al. 1998; Volbracht et al. 1999; Gerhardt et al. 2001; Martinez et al. 2010; Krug et al. 2014). Flow cytometry has also been used for

Table 2 Common endpoints used in neurotoxicity measurements

Read-out	Strength (s)	Weakness (es)
Cell viability		
ATP content, reduction of resazurin or tetrazolium compounds	Simple, can be adapted to HT	In systems with several cell types, results may be hard to interpret, endpoint measurements, limited multiplexing
LDH release	Simple, can be adapted to HT, repeated measurements possible	In systems with several cell types, results may be hard to interpret. Not suitable for repeated dose toxicity. Difficult in long-term culture and with medium change
Direct counting of live/dead cells	Can be adapted to HT, can be multiplexed with proliferation assay	Not suitable for repeated dose measures
Apoptosis (e.g. caspase cleavage, p53 accumulation, mitochondrial dysfunctions, Annexin/PI, phagocytosis)	Can be adapted to HT, multiplexing feasible	Often not suitable for repeated dose measures (exception: SeaHorse)
Morphological or structural endpoints		
Neurite outgrowth and stability	Differential effects of neurites and axons can be quantified	Not suitable for repeated dose toxicity
Functional or subcytotoxic endpoints		
Formation of reactive oxygen species	Amenable to HT	Interpretation of results may be difficult
Cell migration	Amenable to HT	Interpretation of results may be difficult
Changes in differentiation pattern	Can be used in HT setting	Interpretation of results may be difficult
Spontaneous electric activity	Cultures can be continuously monitored	Low throughput, some model systems have low electric activity, expensive
Mitochondrial transport and cytoskeletal integrity	Early detection of cytotoxic effect	Low throughput
Calcium influx	General key event, low- and high-throughput methods available	Difficult interpretation
Cell membrane potential	General key event. High throughput possible by using fluorescent indicators	Difficult interpretation.
Glial activation	Amenable to HT	Results can be hard to interpret in mixed cultures
Myelination	Rich information due to abundant cell–cell interaction and the presence of several cell types	Complex read-out (e.g. changes in gene expression)
Network formation and synaptogenesis	Amenable to HT, highly relevant	Needs exploration of sensitivity
Gene expression profiles	A wide array of mRNA and protein biomarkers may be identified	Expensive, extensive data analysis, difficult interpretation
Neurochemical targets (receptor activation, enzyme activity, ion channel function, neurotransmitter uptake, etc.)	Primary target of test compound can be identified (the molecular initiating event). HT applicable methods	Some methods require radioactively labelled substances.

apoptosis detection of annexin V-stained gently dissociated cells (Sendrowski et al. 2013; Gao et al. 2014).

Cell proliferation may be measured by incorporation of thymidine analogues BrdU or EdU (Mundy et al. 2010). Alternatively, proliferation may be deduced (indirectly) from altered cell numbers (as determined by measurement of DNA, protein, or viability endpoints (ATP; resazurin, LDH)). Cell proliferation and apoptosis have been assessed in the same well in a high-content setting (Culbreth et al. 2012), e.g. in the ReNcell CX immortalized human neuronal progenitor cell line and the mouse cortical neural stem (mCNS) cell line. For this purpose, cell proliferation (BrdU incorporation) was measured in parallel with cleaved caspase-3 and accumulation of p53 as apoptosis markers. Such assay combinations have also been used to evaluate the suitability of cell platforms for neurotoxicity screening (Tong et al. 2016).

Neurite outgrowth and stability

Developmental neurotoxicants may inhibit the extension of axons, dendrites, or both (Lein et al. 2007a). The earliest high-throughput assay for detecting chemicals influencing neurite outgrowth (Radio et al. 2008) used Neuroscreen-1

cells, a commercial subline of the PC-12 rat neuronal cell line. The assay was based on immunostaining for β-III-tubulin to visualize the cellular processes. The scoring algorithm distinguished viable cells from dead cells and neurites from somata. Similar principles have been established earlier to quantify neurite damage in complex human neuronal cultures, and to distinguish this from death of the whole cells (Lotharius et al. 2005).

In a similar approach, the neurite outgrowth of human mesencephalon-derived LUHMES cells was monitored. The LUHMES cell line is a tet-off v-myc conditionally immortalized cell line that stops proliferation and starts differentiation to postmitotic dopaminergic neurons upon the addition of tetracycline. In this assay, neurite area and viability of the cells were assed which allowed specific identification of neurotoxicants by comparison of the EC_{50} values for viability and neurite growth (Stiegler et al. 2011; Krug et al. 2013a).

Beside neurite outgrowth, a further read-out can be to monitor degeneration of already formed neurites (Krug et al. 2013a). The human neuroblastoma SH-SY5Y cell line can be used for both neurite outgrowth studies (Frimat et al. 2010; Bajinskis et al. 2010) and neurite degeneration assays (Forsby 2011), simply by quantifying the number of neurites per cell. For instance, acrylamide-induced neurite degeneration in retinoic acid-differentiated SH-SY5Y cells correlates well with acrylamide-induced neurological adverse effects in vivo (DeJongh et al. 1999). Instead of time-consuming tubulin staining, many modern neurite assays in SH-SY5Y or LUHMES cells use calcein staining of live cell structures and HTS imaging as endpoint (Krug et al. 2013a).

Note that differences can be observed between toxicants acting on axons or dendrites, as several chemicals have opposing effects on axonal and dendritic growth (Howard et al. 2005; Lein et al. 2007b; Yang et al. 2014).

A specific application of neurite assays is the field of chemotherapy-induced peripheral neuropathy. The damage to human neurons generated from pluripotent stem cells has been quantified (Hoelting et al. 2015; Wheeler et al. 2015). This example further corroborates the usefulness of further image-based applications for neuronal development studies or neurotoxicity screening applications.

Formation of reactive oxygen species (ROS)

Formation of ROS is a common sign for cellular stress. A major internal source of ROS is the mitochondrial electron transport chain. Since neurons generate most of their energy via the mitochondrial pathway, they have a particularly high need to control intrinsic ROS formation. Common ways to quantify oxidative stress within cells are the measurement of glutathione (Schildknecht et al. 2009, 2011; Pöltl et al. 2012; Barayuga et al. 2013; Pak et al. 2015), of oxidative stress-responsive transcription factors (e.g. Nrf-2) (Prasad et al. 2013), or the quantification of oxygen radicals with colorimetric or fluorescence-based probes (Krug et al. 2014; Pak et al. 2015).

Cell migration

Neural crest cells (NCC) are formed during vertebrate development from the border of the neuroectodermal plate, and they are vital for generation of facial structures and the entire peripheral nervous system. Disturbed NCC function can be linked to a large proportion of congenital birth defects. A NCC migration assay has been developed to study chemical-induced impairment of neural crest function using NCC cells derived from hESCs or iPSCs (Zimmer et al. 2012, 2014; Dreser et al. 2015). The assay involves counting NCCs that migrate into the cell-free space generated either by scratching the cell monolayer or the removal of a spacer. The latter method also allows for high-throughput screens.

In contrast to NCC migration, assays for the migration of central neural precursors use aggregates (Moors et al. 2009), and this requires more sophisticated software for quantification (Gassmann et al. 2012).

Differentiation pattern

The diversity of neural cell types originates from tightly controlled differentiation programs that are guided by morphogen gradients (e.g. retinoic acid) and cell–cell signalling (e.g. notch pathway). Therefore, especially in the field of DNT, it is of high importance to test whether the differentiation pattern might be disturbed upon exposure to a toxicant. Most published test systems study the differentiation from pluripotent or lineage-committed stem cells to neural cells. The changes in the differentiation pattern are assessed by monitoring marker gene expression or the complete transcriptome over a certain differentiation period (Kuegler et al. 2010; Zimmer et al. 2011a; Balmer et al. 2012; Robinson and Piersma 2013; Krug et al. 2013b; Smirnova et al. 2014a; Waldmann et al. 2014; Shinde et al. 2015; Rempel et al. 2015). For the data interpretation, it is important to consider that changes in gene expression in such models may be caused either by an acute effect of the chemical on a biological process, e.g. signalling pathway, or by impaired differentiation. Instead of transcriptome analysis, also other high-content methods may be used (proteomics, metabolomics, multicolour immunostaining). For instance, the quantification of endogenous metabolites after teratogen exposure could be used to predict human developmental toxicity (West et al. 2010; Kleinstreuer et al. 2011).

Spontaneous activity of neural networks

The ultimate function of the nervous system is the formation of neural networks that transmit and store information. Electrical communication between neurons can be measured by complex electrophysiological methods that are time-consuming and require highly skilled operators. A more recent development of multielectrode arrays (MEA) that measure non-invasively extracellular electrical fields of neurons grown on them. This allows assessment of spontaneous or elicited electrical activity of neuronal circuits in vitro.

A well-controlled study explored whether signals from a MEA may be a useful endpoint for neurotoxicity testing. Mature neuronal networks were established from cultured rat cortical neurons. It was found that the network mean firing rate (MFR) was a useful endpoint for detecting neurotoxic substances (Defranchi et al. 2011).

The next step towards high-throughput application of MEA was taken when effects of a training set of 30 environmental chemicals and drugs on the spontaneous network activity of primary cortical neuronal cultures were measured in 12-well MEA plates. It was confirmed that MFR was a sensitive endpoint (McConnell et al. 2012). Nowadays, the screening standard is to use 24-well MEA plates (Vassallo et al. 2016), and the method should be adaptable for use with human stem cell-derived neurons in a medium- to high-throughput setting.

From a mechanistic point of view, it is interesting to combine the MEA endpoints with other information-rich technologies. For instance, 12 central nervous system-relevant drugs with known toxicity profiles have been tested in an organ-in-a-dish model. Metabolomics provided a quick read-out of changes in cellular physiology and showed changes affecting the metabolism of neurotransmitters. The observed metabolomics changes also translated to changes in activity patterns, as measured by MEA. The MEA results provided 'fingerprints'" characteristic of the different classes of drugs, making MEA a promising early-predictive and sensitive tool for in vitro neurotoxicity testing (Schultz et al. 2015).

Mitochondrial transport

As we mentioned in Chapter 2, neurons are highly dependent on the mitochondrial respiratory chain to maintain their energy levels; thus, they are very susceptible to toxicants affecting mitochondrial function. Transport of mitochondria to the areas of high energy demand is particularly important for neurons due to their long processes. Disturbances in mitochondrial fission and fusion, mitophagy, and motility have been associated with neurodegeneration and might therefore be sensitive indicators for neurotoxicity (Pham et al. 2012; Simcox et al. 2013). Several high-content imaging protocols assessing mitochondrial movement have been established for a diversity of cells, which might be also applicable to neurons (Koopman et al. 2006; Mitra and Lippincott-Schwartz 2010; Leonard et al. 2015). For instance, it has been shown that dopaminergic neurons react with a change in motility already 6 h after exposure to MPP^+, more than 24 h before cell death occurs (Schildknecht et al. 2013).

Calcium influx and cell membrane potential

An important common key event in neurotoxicity is the dysregulation of intracellular calcium signalling. This may be measured by the use of calcium-binding fluorescent probes (Leist and Nicotera 1998; Lock et al. 2015) under basal conditions (e.g. spontaneous calcium oscillations), or by using well-controlled stimuli, such as cell depolarization by exposure to veratridine or high concentrations of K^+ ions, or by stimulation with specific neurotransmitter receptor agonists. Furthermore, it is possible to detect differences in calcium storage and release in response to a toxicant (Abushik et al. 2014; Sisnaiske et al. 2014; Hausherr et al. 2014; Meijer et al. 2015).

Any change in ion distribution over the cell membrane will result in altered cell membrane potential (CMP), i.e. depolarization or hyperpolarization. Monitoring the CMP by using fluorescence indicator dyes is a cheap and robust approach that can be adapted relatively easily for high-throughput screening (Galofré et al. 2010; Gustafsson et al. 2010). The choice of cell model is essential in these studies since the target(s) that are affected by the toxicant must be expressed (Forsby et al. 2009).

Gliosis

Glial cells comprising astrocytes, oligodendrocytes, microglia, and NG2 glia are important not only for the maintenance and physical support of neurons but also for the development of the nervous system. The proliferation of glial cells (gliosis) is a common feature of central nervous system damage, and alterations in glial function can affect neuronal proliferation, differentiation, and regeneration (Aschner et al. 1999; Kuegler et al. 2010; Kadereit et al. 2012; Burda and Sofroniew 2014; Anderson et al. 2016).

Glial fibrillary acidic protein (GFAP) and related glial biomarkers are used in neurotoxicity studies to distinguish glial cells from neurons (Monnet-Tschudi et al. 2000; O'Callaghan and Sriram 2005; Monnet-Tschudi et al. 2007; Sandström von Tobel et al. 2014). A high-content analysis method for gliosis induced by treatment

with neurotoxicants has been developed on the basis of co-cultures of rat neurons and astrocytes (Anderl et al. 2009). For such studies, the availability of a pure, defined, and well-characterized astrocyte population is highly desirable (Kuegler et al. 2010, 2012; Schildknecht et al. 2012; Kleiderman et al. 2015). Murine microglia and astrocytes have been extensively characterized for inflammation studies and neuronal co-cultures (Falsig et al. 2004a, b, 2006, 2008; Lund et al. 2006; Efremova et al. 2015, 2016). Some studies on the human counterparts are available, but the characterization is still lagging considerably behind (Pei et al. 2015; Palm et al. 2015).

Myelination

The myelin sheath, built by oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system, is wrapped around the axons of neurons. It is the key structure enabling a fast and efficient flow of electrical impulses along axons, and therefore is essential for the functioning of the nervous system (Bunge 1968). The role of myelinating cells goes far beyond electrical insulation: they also modulate axonal growth, neuronal survival, and neuronal metabolism (Miller 2002; Nave and Salzer 2006).

Studies in re-aggregating rodent brain cell cultures have shown that interactions between the different cell types (neurons, astrocytes, and oligodendrocytes) provide an environment facilitating neuronal differentiation, which leads to the development of synapses and myelin (Guentert-Lauber et al. 1985). Cellular composition of the aggregates is usually quantified by changes in gene expression (Zurich and Monnet-Tschudi 2009; Forsby et al. 2009) or by cell-type-specific enzyme activity (Monnet-Tschudi et al. 2000) or by success of differentiation into oligodendrocytes (Schreiber et al. 2010). Other studies use primary dorsal root ganglion and Swann cells for myelination assays, and lipid stains or marker gene expression are used as endpoints (Stettner et al. 2013).

As defects or impairment of myelination have been connected to neuronal diseases such as multiple sclerosis, psychiatric diseases, and diverse peripheral neuropathies, several hESC/iPSC-based in vitro protocols have been developed to generate oligodendrocytes in order to model their interaction with neural and glial cells. The resulting cells have been shown to form multilayered myelin sheaths around axons in co-cultures (Preynat-Seauve et al. 2009; Moors et al. 2009; Sundberg et al. 2010; Martinez et al. 2012). In vitro test systems that cover all three cell types of the brain, using myelination as an endpoint to assess neurotoxicity in hESC/iPSC-based in vitro systems, are still challenging, and good models need to be developed.

Network formation and synaptogenesis

Synaptogenesis involves the formation of elaborate presynaptic structures and the recruitment and stabilization of neurotransmitter receptors in the postsynaptic membrane. Synapses are readily and abundantly formed in primary neuronal cultures, e.g. based on hippocampal or cortical cells. hiPSC-derived neurons also show synaptic vesicles, increased expression of synapse-associated markers, such as the presynaptic proteins synaptophysin, synaptobrevin, SNAP25 and syntaxin as well as the postsynaptic proteins PSD95, shank, and homer (Araque and Navarrete 2010; Brennand et al. 2011). Formation of functional synapses occurs considerably later and with lower efficiency than the expression of the respective proteins. Therefore, the ultimate test for synaptogenesis is based on electrophysiological features, like coordinated network activity.

A surrogate endpoint for synaptogenesis is based on the assumption that pre- and postsynaptic proteins should co-localize at synapses. On this basis, synapse formation assays have been developed that detect punctate staining of synapsin in close apposition to dendrites in a high-content imager (Harrill et al. 2011).

Such assays have shown that co-culture with astrocytes favours synaptogenesis of neurons (Roqué et al. 2011; Kleiderman et al. 2015). All methods to measure the aforementioned endpoints are well established for 2D cultures and need to be optimized and adapted for emerging 3D cultures (Andersen et al. 2013).

Analytical methods for neurotoxicity and developmental neurotoxicity screening

In this section, we will review analytical methods suitable for neurotoxicity and developmental neurotoxicity screening, particularly those that are amenable to “high-throughput” methods that typically handle cells in multiwell plates and involve automated scoring of the results (Table 3).

Spectrophotometric, fluorimetric, and luminometric endpoints

The earliest and most frequently used techniques for high-throughput screening are based on alterations in light absorbance, fluorescence intensity (FI), or light emission. Common cell viability assays, such as the MTT-reduction or neutral red uptake tests, are measured photometrically, and resazurin reduction (also called AlamarBlue) is measured by quantification of absorption or FI. As the detection of fluorescence is more sensitive, it is used for more ‘difficult’ measurements, such as cellular calcium ion concentrations, ROS formation or membrane potential of the

Table 3 Summary of discussed analytical methods for NT and DNT testing

	Continuous recording	High throughput	High-content information	Strength (s)	Weakness (es)
Classical techniques (FACS, ICC)	No	No	Partially	Well established	Cost-/labour-/time-intensive
Impedance measurements	Yes	Yes	No	Inexpensive	Only cell numbers can be directly quantified (live/death quantification)
High-content imaging	Yes (with low time resolution)	Limited	Yes	Many cellular features can be analysed	High data load, data analysis very complex
Spectrofluorometry	Yes	Yes	No	High throughput, possible, specific for general functional mechanisms	Possible interference between the fluorescent indicator and test compounds
Multichannel parallel microscopy cytometer	Yes	Yes	Limited	Highest throughput of all techniques	Sparse 1D images may cause ambiguous results
Transcriptomics	No	Yes	Yes	Comparison of whole genomes possible	Costs; expression patterns for cytotoxicity of different cell lines and drug classes yet to be defined
Metabolomics	No	Yes	Yes	Comparison of whole metabolome possible	Metabolome patterns for cytotoxicity of different cell lines and drug classes yet to be defined

plasma membrane or the inner mitochondrial membrane. Absorbance or FI is also used for many enzyme activity assays, such as the one for lactate dehydrogenase (LDH, used for cell viability estimates) or for acetylcholine esterase (AChE, used to evaluate the potential of toxicants to inhibit this pivotal toxicity target). Moreover, most immunodetection assays (e.g. for cytokines, neurotransmitters, or neuron specific enolase) use such endpoints. Alternatively, such assays sometimes use light emission (based on luciferase reactions with ATP) as endpoint (most frequently applied for estimations of the ATP concentration or for gene reporter assays. All these three analytical endpoints (Absorbance, FI, light emission) can be monitored in 96-well, 384-well, or 1536-well plates with equipment available in most standard screening facilities.

Impedance measurement

Impedance monitoring is a label-free and non-invasive approach for cytotoxicity testing that provides real-time read-outs. The salt concentration and temperature of the solution, as well as the attachment of cells to the electrode, affect electrode impedance at the electrode/medium interface. The more cells cover the electrodes, the larger the electrode impedance is, but the quality of the attachment also crucially influences impedance values. The gold electrodes are integrated into a microelectrode array in the bottom of 96-multiwell culture dishes. The output is a dimensionless parameter termed the cell index that reflects changes in cell number, morphology, adhesion, and viability caused by cytotoxic compounds (Diemert et al. 2012).

An advantage of impedance measurements is that cell index changes can be accurately detected in a wide range of cell densities, while for example MTT signals are cell number dependent, requiring the optimization of the number of seeded cells for each cell line. Moreover, impedance measurement allows continuous real-time monitoring and can inform about the kinetics of cell death as opposed to endpoint analysis like MTT assays. The possibility to scale-up impedance measurements to medium- or high-throughput screens has already been demonstrated (Hou et al. 2014).

One disadvantage of impedance measurements is the perturbation of the cell index after medium change or drug application due to disturbances in medium temperature. Therefore, this technology is not well suited for short-term monitoring of <3–4 h. Moreover, currently available technology does not allow quantification of neurite networks, probably due to the limited spatial resolution of the gold electrodes used.

Electrophysiological screens

Disruption of neuronal excitability produces substantial and rapid disruption of nervous system physiology, and often precedes cell death and other biochemical or morphological changes. Current in vitro assays based on biochemical and morphological changes are not optimized for detecting this type of toxicity. In this regard, electrophysiological

techniques such as patch-clamp (Bosca et al. 2014) and MEAs have several advantages over biochemical methods for the assessment of in vitro neurotoxicity (Köhling et al. 2005).

Developed by Neher and Sakmann over 40 years ago, patch-clamp has remained the gold standard for the study of ion channel activity at the single molecule level (microscopic current) and at the cellular level (macroscopic current) (Neher and Sakmann 1976).

The development of automated patch-clamp (APC) systems allows us today to collect large amounts of data in a short period of time. High-throughput patch-clamping can be very useful during the initial stages of pharmaceutical lead compound development (also known as "first screen") because these steps are usually focusing on a single molecular target (i.e. ion channel) at a time. However, because current APC devices require the use of cells in suspension, they are not suitable to study ion channel activity in cells within neuronal networks.

In contrast to APC, MEAs can be used to monitor pharmacological effects in cellular networks (Johnstone et al. 2010). MEA recorded field potentials provide information on cellular network activity and plasticity, which are critically important for CNS homeostasis. Another advantage of this system is its ability to predict toxicity without prior knowledge of a chemical's mechanism of action since these agents may affect functionality by changes in receptor modulation, or metabolic disruptions (Novellino et al. 2011). Furthermore, in contrast to patch-clamp and APC, MEAs are suitable for long-term recordings.

High-content imaging

High-content imaging (HCI) refers to automated (multicolour) fluorescence microscopy in a high-throughput setting (van Vliet et al. 2014). Information gained by HCI includes changes in fluorescence intensity and distribution, cell morphology, and cell movement. Although many HCI studies involve immunocytochemical staining of fixed cells, continuous monitoring of live cells is also possible. HCI can provide detailed information on cell movement, changes in cell morphology, or intracellular distribution of proteins or inorganic ions that cannot be obtained with impedance measurements, for example.

Automated sample positioning and autofocusing are essential features for fast data acquisition of such image-based methods, but the automated and yet accurate analysis of the enormous amounts of HCI data acquired is even more important. For neurocytotoxicity HCI testing of compound libraries containing several thousand compounds on several cell types/lines in parallel, the expected image numbers will be extremely high (up to billions), and automated data processing pipelines are mandatory to handle the amounts of information more efficiently. A number of automated image analysis tools have been developed to process images and to analyse features of neurocytotoxicity [see (Billeci et al. 2013) for an overview of tools], we would mention NeurphologyJ (an ImageJ plugin), HCS-Neurons, NEMO, and Cellprofiler here (Carpenter et al. 2006; Ho et al. 2011; Billeci et al. 2013; Charoenkwan et al. 2013; Dreser et al. 2015). HCS-Neurons aim at analysing the groups of neurons (multineuron images), and NEMO provides the user with a unique function enabling the analysis of sequences of time-lapse images.

A few technical issues still limit high-throughput application of HCI: (1) acquired data volumes require an appropriately sized data storage and processing infrastructure; (2) analysis needs considerable manual adjustments to avoid background noise, which is time- and therefore cost-intensive; (3) assay development is challenging and time-consuming; and (4) the speed of data acquisition still needs improvement.

Multichannel parallel microscopy cytometry

Parallel microscopy cytometry (PMC) combines a number of advantages of classical single-channel flow cytometry devices and HCI systems (Ehrlich et al. 2011; McKenna et al. 2011): PMC allows parallel recording of 384 channels as compared to a conventional single-channel FACS instrument and, at the same time, provides higher detection sensitivity through increased signal-to-noise ratios. Currently, PMC allows for the same count rates as high-end FACS with even increased rare-cell detection capability.

Compared to HCI systems, focusing problems are eliminated by flowing the samples into focus. This drastically reduces the time required to image multiple cells (that would require moving the stage in a HCI system). As the developers claim, a PMC system with integrated robotic sample loading can read 384 samples in 6 min in a binary assay (distinguishing only positive and negative events), whereas only about 10 samples can be processed using a single-channel FACS instrument in the same time (Ehrlich et al. 2011).

While HCI systems work with 2D images, which are relatively large data sets, PMC produces multicolour 1D imaging. Recording data as highly economic 1D images significantly reduces the data load and allows real-time analysis with the corresponding algorithms (Ehrlich et al. 2011; McKenna et al. 2011). The results showed good proficiency in protein aggregation and NFkB nuclear translocation assays, but the developers also indicate the disadvantage of sparser images leading to potentially ambiguous results.

Future developments and improvements of PMC systems may include achieving ultra-high count rates by improved digitization electronics and consequently further

increased sample throughput so that up to two 384-well plates per minute can be processed. Cell sorting could be added to the PMC system to allow separated collection of samples for further downstream analysis (e.g. qPCR or mass spectrometry). Eventually, PMC may be a promising tool to study very fast aspects of biological kinetics due to its high temporal resolution combined with high-content information acquisition capability.

Transcriptomics

Characterization of mRNA changes has become a major endpoint to assess toxicant effects in stem cell systems or developing zebrafish (Kuegler et al. 2010; Robinson and Piersma 2013; Smirnova et al. 2015b; Schwartz et al. 2015). One of the publications resulting from the pan-European ESNATS research project (Embryonic Stem Cell-based Novel Alternative Testing Strategies) investigated whether it is feasible to use transcriptional profiling for developmental neurotoxicity testing (Krug et al. 2013b). A large overlap was found between the transcription factor binding sites (TFBSs) enriched in response to different drug treatments in different test systems, suggesting that it may be possible to identify a set of common cytotoxicity TFBSs as well as TFBS signatures specific for certain chemicals. For example, mapping of putative TFBSs led to the identification of new pathways relevant to dopaminergic neurotoxicity (Krug et al. 2014; Maertens et al. 2015). Various studies also showed that transcriptomics data is concentration dependent (Piersma et al. 2011; Waldmann et al. 2014), and that they are suitable for biomarker identification and blinded classification of compounds (Rempel et al. 2015; Pallocca et al. 2016). Theunissen et al. found that whole genome gene expression profiling combined with annotation of enriched genes based on their gene ontology class provided a more sensitive read-out to classify toxicants than morphological changes (Theunissen et al. 2012).

microRNA profiling (miRNomics)

microRNAs (miRNAs) are small non-coding regulatory RNA molecules, which regulate expression of more than 60 % of genes by binding to the 3′ UTR of mRNAs and repressing their translation. It is known that miRNAs play a significant role in brain development and function (Li and Jin 2010). They are also involved in cellular responses to environmental stress, including chemical exposure (Smirnova et al. 2012).

Several studies have addressed the role of miRNAs in neurotoxicology (Huang and Li 2009; Miranda et al. 2010; Tal and Tanguay 2012; Pallocca et al. 2013; Smirnova et al. 2014a; Meganathan et al. 2015). As miRNAs are phylogenetically conserved, extrapolations between different species are possible. Thus, miRNA profiling, together with identification of the predicted mRNA targets, may have stronger predictive value for the mechanism of action than mRNA alone, as miRNAs were established as crucial developmental regulators that mark developmental timing and cell specification [reviewed in (Bartel 2004)].

Recently, miRNAs have been detected in biofluids (Weber et al. 2010). These circulating miRNAs are much more stable than mRNA and can be used as biomarkers for a variety of diseases (Wang et al. 2013) and organ toxicity. Identification of miRNAs involved in the response to chemicals in the supernatant in in vitro neural models can give indications for further research on potential circulating biomarkers.

Metabolomics

Similar to the aforementioned approaches quantifying gene expression, small molecules secreted by cells, or being present within cells, can also be considered as possible biomarkers to trace toxicity-induced cellular changes (Bouhifd et al. 2013). Metabolic endpoints mirror physiological states as well as the adaption of cells to changing conditions.

Several publications employed mass spectrometry to analyse the metabolome of in vitro models before and after toxicant application. Cezar et al. measured small molecules secreted by hESCs and neuronal progenitors derived from them. They detected valproate-induced differences that point to key neurobiology pathways (Cezar et al. 2007). West et al. (2010) published a study investigating developmental cytotoxicity that provided evidence that teratogenic drugs altered the metabolome in hESCs (West et al. 2010). Their work showed a correlation between teratogenicity and the ratio of the levels of secreted arginine and asymmetric dimethylarginine (ADMA). In another study, the effects of ethanol on hESC-derived embryoid bodies, neural progenitor cells, and neurons were investigated in the context of foetal alcohol spectrum disorders (Palmer et al. 2012).

The metabolomics strategy may identify small molecule biomarkers for preclinical safety testing of chemicals and toxicants (Ramirez et al. 2013). Moreover, if the secretome in the media of in vitro models is analysed (and not the metabolome of cell lysates), not only fixed timepoint analysis, but also continuous tracing of drug-induced changes over longer periods will be possible. However, it should be noted that especially untargeted metabolomics (i.e. metabolomics without the prior definition of measured substances by reference materials) comes with many challenges, especially metabolite identification, and issues for standardization and quality assurance (Bouhifd et al. 2015a). We also need to be aware that metabolomics measurements

may cover only a subset of metabolites, depending on the extraction method and choice of instruments.

Strategic considerations for neurotoxicity testing

Integrated testing strategies

It is unlikely that any single model—even in combination with high-content read-outs—can provide information sufficient for a comprehensive assessment of phenomena as complex as (developmental) neurotoxicity. Therefore, various tests will need to be combined. Building such a battery of tests has also some shortcomings, such as the accumulation of false-positive results. As a solution, integrated testing strategies (ITS) have been proposed, where various information sources are combined in an optimized algorithm (Hartung et al. 2013a; Leist et al. 2014; Rovida et al. 2015; Bal-Price et al. 2015a). The term ITS is now often replaced by IATA (integrated approaches to testing and assessment) as favoured by OECD.

This approach also comes with a number of challenges: a number of tests needs to be available simultaneously and in one place to allow testing of a given substance; the costs of testing multiply (and already now alternative methods are often not really competitive to their in vivo counterparts); quality-control and validation of such complex decision trees is also extremely difficult.

To structure the information, the adverse outcome pathways concept has been suggested to be used as a basis for ITS development (Basketter et al. 2012; Leist et al. 2014; Tollefsen et al. 2014). Progress towards test batteries (e.g. in the A-cute-Tox, ESNATS, and ChemScreen projects) and AOP (Vinken 2013; Smirnova et al. 2014b; Bal-Price et al. 2015b; Becker et al. 2015) is continuously being made.

Multiomics integration for identification of 'pathways of toxicity'

Individual omics and HCI technologies create data-rich situations, now often called 'big data', and the challenge lies in making big sense of these big data. The high number of parameters measured, e.g. the 27,000 human genes in modern transcriptomics approaches, offer too many opportunities for overfitting, given the small numbers of measurements in comparison. Therefore, we will always find genes which show dose or time dependence in a given experiment. This calls for reduction in dimensionality, and tracing the signatures of changes back to the underlying mechanisms (Hartung and McBride 2011). The situation is further complicated by considerable noise in these measurements. Some of the noise originates from the biological models, but also the technologies themselves have often variances relevant to the effect sizes observed. This led to the suggestion of overlaying different omics approaches to separate signal and noise in the Human Toxome project (Bouhifd et al. 2015b).

Advanced technologies and bioinformatics allow us to use multiomics techniques including (toxico)genomics, epigenomics, proteomics, transcriptomics, metabolomics, and miRNomics as an integrated approach for a systematic analysis of biological changes. This makes it feasible to study systems biology in terms of development, disease, pharmacology, and toxicology. Advancing bioinformatics tools such as gene clustering and deduction of gene, miRNA and metabolite correlation networks based on multiomics data can bridge the gap between molecular initiating events and adverse outcomes for a better understanding of the mechanisms underlying neurological disease development as well as DNT.

"In vitro" testing for functional impairment of the nervous system

As illustrated in Chapter 3, neurotoxicity testing is done according to different test guidelines, depending on the life stage of interest. The design of a neurotoxicity study is fundamentally different from a DNT study. This is exemplified by costs for the latter being around an order of magnitude higher than that of standard neurotoxicity testing. As the studies for current regulatory use are so different, it makes sense to also separately discuss the respective in vitro assays, and how to use them to eventually substitute or at least reduce in vivo testing.

Neurotoxicity testing is mostly concerned with toxicity to the nervous system of the adult organism. Here, two major types of toxicity need to be distinguished:

Acute toxicity

Getting manifest after one or few doses, and being identified in 28-day studies. Acute toxicity typically arises from acute functional disturbances, e.g. by modulation of ion channels, neurotransmitter levels, or neurotransmitter receptors. In some rare cases, acute toxicity may also be due to structural damage to a defined neuronal subpopulation. For instance, MPTP may damage dopaminergic neurons of the *Substantia nigra*. This type of damage would not be identified on the histopathological level in a guideline study, because the *Substantia nigra* is not routinely sectioned, and since the type of damage would not be apparent with standard histological methods. Thus, in both cases (functional damage to neurotransmission and pinpointed structural damage), the adverse effects would most likely be detected as functional endpoints. They would be observed by altered behaviour in the own cage or in open-field testing, or they would get evident by more dedicated neurofunctional testing, e.g. for motor

control. The goal of in vitro methods is to identify such acute functional disturbances of the nervous system with similar or better sensitivity, and this must determine the design of tests and the assembly of test batteries.

Evidently, the type of information delivered by an in vitro battery will be different from the information observed during human exposure. For instance, drowsiness, headache, confusion, or reduced cognitive capacity will not be directly measurable in vitro. However, this information is also not directly available from animal studies, either. There is no direct correlation to an open-field test or a home cage observation in human pathology, but experience has taught toxicologists what inferences can be made. The important point is that all (or most) relevant adverse effects are observed, and that these can be quantified to a certain extent so that safe exposure levels can be derived.

The same reasoning applies also to in vitro test methods. It is not clear whether a disturbance in mean firing rate in a MEA assay reflects drowsiness or another symptom, but it gives an indication of a potential hazard occurring at a certain active site concentration of the test chemical. For testing of acute neurotoxicity, therefore following exemplary test battery could be envisaged: acute structural damage would be observed in models of different neuronal and glial subpopulations, and the threshold of toxicity would be compared to other cell types to determine whether the effect is neurospecific. Functional impairments would be screened for in assays of calcium signalling, membrane potential measurement, and spontaneous (or elicited) network activity, preferentially based on different types of neurons. Ideally, such in vitro tests in the narrow sense would be complemented by tests using simple model organisms, such as zebrafish, that allow more classical sensory and motor testing.

Chronic toxicity

Requiring long-term dosing is observed in 90-day or longer studies. A typical manifestation of chronic toxicity is peripheral neuropathy triggered by industrial chemicals such as hexane or acrylamide, by some carbamate pesticides or by chemotherapeutics. CNS chronic toxicity may arise from cycasin or from beta-*N*-oxalyl amino-L-alanine (L-BOAA) in food, and it may also be a consequence of low-level exposure to the pesticide rotenone. In the latter case, animal studies indicate damage of *Substantia nigra* dopaminergic neurons as main adverse effect (Johnson and Bobrovskaya 2015). Such chronic toxicity is sometimes hard to detect by regulatory animal studies, and determining the relevance of the data to man is highly complex.

For in vitro studies, it is not yet clear how well this area is covered. Although the same endpoints can evidently not be measured, the modern technology applied to in vitro tests might nevertheless allow hazard identification. This hope is based on two assumptions: First, the multiomics endpoints might reveal cellular changes that are not obvious from viability or morphology studies, but are nevertheless relevant for prediction of functional impairment. In fact, such very early changes may be observed after relatively short exposure (compared to a 90-day study), and still predict hazard after prolonged dosing. Second, as more and more AOPs become available and validated, more KE relevant to chronic toxicity become known. Instead of testing for apical endpoints (e.g. histopathology or behavioural change), in vitro tests can test for the disturbance of such key events and, based on the AOP framework, infer the outcome on the level of the organism. For instance, compounds may lead to functional impairment of mitochondria, long time before histopathological damage is observed in the *Substantia nigra*. Thus, testing for the KE 'mitochondrial dysfunction' within a test battery would yield data that classify rotenone as a potential chronic neurotoxicant.

DNT

Toxicity to the developing nervous system is fundamentally different from adult neurotoxicity. Also, in this area or regulatory testing, histopathological endpoints are combined with functional tests. In vitro tests must be able to deliver a similar type of hazard information. At present, the concept of how this may be achieved is based on the assumptions that (1) functional changes observed in man or animals are due to a toxicity endophenotype (Kadereit et al. 2012; Smirnova et al. 2014b; Bal-Price et al. 2015b), i.e. a change in neural connectivity in at least one region of the nervous system, (2) that such a change must result from the disturbance of at least one fundamental neurodevelopmental process (e.g. neurite growth or neuronal differentiation), and (3) that such processes can be modelled by in vitro tests.

Some of the tests presented in this review would form a part of a test battery to assess all relevant neurodevelopmental processes to investigate whether one of them is disturbed by chemicals. Hazard definition would then need to be adapted to the types of endpoints produced by in vitro testing. A possible bridging may be obtained by the inclusion of model organisms (e.g. *C. elegans*, *Drosophila* or zebrafish) that allow direct observation of neurodevelopmental processes, their change by chemicals and the final consequence for the toxicity endophenotype and organisms function.

Considerations of concentrations and timing for toxicity testing endpoints, in order to separate adverse effects (toxicity) versus adaptive changes

Concentration

For all in vitro assays, it is difficult to distinguish between changes that are linked to adverse effects in vivo, and

alterations that are only adaptive or counter-regulatory (Blaauboer et al. 2012).

An overall solution to this challenge will be a major issue for the future, but a few points deserve immediate attention and action. The first and foremost is 'concentration'. The questions of specificity and adversity cannot be linked to compounds as such, but only to a 'compound at a given concentration' (Waldmann et al. 2014; Daston et al. 2014). Although this appears trivial, it has hitherto been little considered, when specificity and sensitivity of an assay have been evaluated. Even some official validations by EU institutions, such as the one of the embryonic stem cell test (EST), did not include this consideration in the suggested prediction model. Also, most published toxicological screens have up to now been performed at fixed compound concentrations that are not related to the pharmacological potency of the compounds screened. A change in this practice has been suggested for the ESNATS test battery (Zimmer et al. 2014; Pallocca et al. 2016), for which starting concentrations for testing have been based on a biological/mechanistic rationale. Also, the European ChemScreen program seriously addressed this issue, and very high-throughput screens of the American Tox21 program automatically cover wide concentration ranges (Tice et al. 2013; Attene-Ramos et al. 2013). It also should be noted that for many omics studies in the field of DNT, the chosen concentration is anchored on a biological effect (e.g. maximum non-cytotoxic concentration) (Robinson and Piersma 2013; Krug et al. 2013b; Rempel et al. 2015).

In practice, the task of determining which concentrations are meaningful and correspond to in vivo effects are not trivial, and they can be quite difficult to determine (Piersma et al. 2011; Westerink 2013). A future help for the field would be a consensus document suggesting such concentrations for DNT compounds and neurotoxicants based on reverse pharmacokinetic modelling (Bosgra and Westerhout 2015). One of the approaches to define adversity would be based on measuring concentration dependency of many endpoints in the system and relating these dependencies to the concentration known to be associated with adverse effects in vivo (Inglese et al. 2006; Parham et al. 2009). Concentration is also a neglected issue for negative controls, used to evaluate assay specificity. Negative data become only meaningful at certain concentrations. They are sheer nonsense if compounds are used at too low concentrations.

Timing

Another useful approach would be to not only rely on measurements at a defined time point at the end of the incubation, but to follow the temporal evolution of changes for the system as such, and for the system under the influence of test compounds (Dreser et al. 2015).

An analogy is used here to illustrate how this approach is expected to give richer information on adverse effects of test compounds: 'After a panic incident, causing many fatalities in a shopping mall, it is very difficult to reconstruct the initial cause, and the chain of events that led to chaos and loss of lives, based on the position of people afterwards (when paramedics, fire brigade and repairmen have already arrived); it would be easier (and clearer), if the whole time course of events was recorded (filmed)'. Moreover, if reactions to initial stimuli with different intensities (e.g. type and size of a shop fire) would have been known from experience or trials, this would have allowed predictions on when normal evacuation shifts to panic. Exactly that approach, i.e. following events over time and probing controlled disturbances, would be useful for establishing predictive DNT/NT test systems with tool compounds: adversity would be predicted based on the quantification of the dynamics of all the essential events (concentration–time response modelling), and linking them by a mechanistic model.

Conclusions

An enormous number of in vitro test systems and functional endpoints have entered the field in recent years, very much fuelled by the availability of stem cell-derived human neuronal models. It is now important to tailor these assays based on their fitness for purpose (Rossini 2012): Different uses of the cell systems will require different degrees of sophistication; for example, human-on-a-chip approaches are based on complex organotypic cultures to approximate the repertoire of human physiological reactions, and high-throughput tests require simplicity and robustness. The new paradigm of toxicology for the twenty-first century needs complex models for pathway identification and simpler assays for testing the perturbation of any given pathway. The more we know about the underlying mechanisms, the less complexity and more specificity we will seek.

Despite the ethical and regulatory constraints associated with the use of hESCs, they have been utilized, especially for developmental neurotoxicity test establishment. In the future, they will be replaced by hiPSC derivatives for screening purposes and for toxicity prediction in humans. It is expected that models based on iPSC derivatives will drastically reduce the number of animals needed for toxicology studies while improving the quality of the results.

One bottleneck for hiPSC-based toxicity assays today is the lack of protocols to generate sufficient quantities of cells, and of generating neural populations identical to cells found in vivo. In spite of improvements in differentiation efficiency, there is still a need for protocols that provide mature neuronal cells in large numbers at moderate cost

and within shorter time spans. In the meantime primary or immortalized neuronal precursors will continue to provide a good alternative to hiPSC-derived neurons.

A common criticism of using hiPSCs is that they only reflect the genetic background of the person they were generated from. Screening results obtained using hiPSC-derived cells therefore might not represent well the whole population, since certain individuals may react differently to a given compound. Although this is true—not only for iPSCs, but also for hESCs, primary cells, and tumour cells—we should bear in mind that animal experiments are also performed using inbred animals with a very limited genetic background. Genetic diversity of humans can be represented with the use of several cell lines originating from different genetic backgrounds, and might include individual backgrounds where clinical signs of high sensitivity to toxic effects (mostly in cases of drug-induced toxicity) have been detected (Brewer et al. 2016).

DNT has moved into the centre of attention because there is evidence suggesting that environmental chemicals can be linked to the increased number of children showing neurodevelopmental disorders, including lowered IQ, learning disabilities, and autism spectrum disorders (ASD) (Coccini et al. 2006; Grandjean and Landrigan 2006; Landrigan 2010; Smirnova et al. 2014b). As the available information on DNT effects of chemicals is particularly sparse, there is an urgent need for reliable and cost-effective screening methods providing specific DNT information of potentially harmful chemicals. New and already existing high-throughput screening assays in combination with hiPSC-based in vitro models possess a great potential to elucidate certain aspects of neurotoxicity and developmental neurotoxicity at the same time. Many of these methods are amenable to high-throughput screening. This will challenge the current validation paradigm both because such HTS platforms are too rare to allow ring trials, and because direct comparisons with a single traditional animal test are not possible. However, concepts to handle these two problems are emerging (Leist et al. 2012; Hartung et al. 2013b; Judson et al. 2013).

While a lot of technologies for acute neurotoxicity and DNT assays are already available, more work will be required to address issues of chronic neurotoxicity and toxicity arising from chemical interaction with rare targets, not well represented in in vitro models. While the tests presented here have a high value for screening, and indication of potential neurotoxicity hazard, the interpretation of the data and prediction of functional effects in humans are still an open research field. It would be naïve to assume that any in vitro model in isolation can satisfy the information needs for (developmental) neurotoxicity. It is much more likely that integrated testing strategies (ITS) (Hartung et al. 2013a; Rovida et al. 2015) have to be developed, which efficiently combine several such methods including possibly in silico, *in chemico*, and even in vivo assays. It is noteworthy that the concept of ITS is increasingly embraced by OECD (Tollefsen et al. 2014) as integrated approaches to testing and assessment (IATA), expecting that AOPs will allow us to construct ITS/IATA based on pathway knowledge. This review took stock of the emerging building blocks of such test strategies. The discussion on how to combine them to develop a humane and human-relevant approach has only started.

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Compliance with ethical standards

Conflict of interest B.Z.S, M. Lehman, E.N., H.X.A., J.K., and A.D. had been or are involved in a company that produces iPSCs, does neural differentiation, and has a commercial interest in the field of (developmental) neurotoxicity testing and stem cell products. S.N. is married to B.Z.S. T.H. is cofounder of Organome LLC, the licensee of a provisional patent filed by Johns Hopkins University for a human mini-brain mentioned in this paper (inventors David Pamies, Helena Hogberg and Thomas Hartung). L.S. is married to T.H.

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