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Workgroup Report: Incorporating In Vitro Alternative Methods for Developmental Neurotoxicity into International Hazard and Risk Assessment Strategies

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This is the report of the first workshop on Incorporating In Vitro Alternative Methods for Developmental Neurotoxicity (DNT) Testing into International Hazard and Risk Assessment Strategies, held in Ispra, Italy, on 19–21 April 2005. The workshop was hosted by the European Centre for the Validation of Alternative Methods (ECVAM) and jointly organized by ECVAM, the European Chemical Industry Council, and the Johns Hopkins University Center for Alternatives to Animal Testing. The primary aim of the workshop was to identify and catalog potential methods that could be used to assess how data from in vitro alternative methods could help to predict and identify DNT hazards. Working groups focused on two different aspects: a) details on the science available in the field of DNT, including discussions on the models available to capture the critical DNT mechanisms and processes, and b) policy and strategy aspects to assess the integration of alternative methods in a regulatory framework. This report summarizes these discussions and details the recommendations and priorities for future work. Key words: high-throughput screening, in vitro developmental neurotoxicity models, regulatory use, validation. Environ Health Perspect 115:924–931 (2007). doi:10.1289/ehp.9427 available via http://dx.doi.org/ [Online 6 February 2007]

Chemicals present in the environment have a potential impact on neurodevelopment and children’s health. In recent years, much attention has been given to model development and risk assessment procedures for reproductive toxicity, but the specific area of developmental neurotoxicity (DNT) has been relatively neglected in testing and risk assessment studies. Although epidemiologic and animal studies on developmental neurotoxicants have been carried out (Evangelista de Duffard and Duffard 1996), most chemicals in use have been tested scarcely or not at all for DNT. To properly assess the risk of chemicals for human health, data on DNT are necessary and this need is recognized by all stakeholders.

In 1998, the U.S. Environmental Protection Agency (U.S. EPA 1998) published the Health Effects Test Guidelines OPPTS 8706300 on DNT (U.S. EPA 712-C-98-239), and the Organisation for Economic Co-operation and Development (OECD) is currently finalizing a new draft Test Guideline (TG) for DNT (OECD 2006). To support and promote these efforts, this workshop focused on two immediate needs for DNT testing: first, the identification of in vitro and nonmammalian alternative methods that may recapitulate critical aspects of the development of the human nervous system; and second, how results from such alternative methods could be integrated into current in vivo testing strategies and the existing regulatory framework. Our hope is that this approach will decrease the number of chemicals reliant on DNT data solely from in vivo mammalian DNT tests and, consequently, refine, reduce—and maybe partly replace—the need for animal testing. Furthermore, we hope this workshop report will provide the basis for discussion in the expert communities on DNT testing and that such a discussion will identify the best steps forward.

Definition of DNT

Chemicals may adversely affect the nervous system in various ways (Ray 1999). They may perturb commitment of neural stem cells, proliferation of neuronal progenitor cells, cell migration, synaptogenesis, cell death, formation of transmitters and receptors, trimming of connections, myelination, and development of the blood–brain barrier (BBB). Impairment of the nervous system can lead to a variety of health effects such as altered behavior, mental retardation, and other neurodevelopmental disabilities and diseases (Li et al. 2005; Olney 2002; Rodier 1995).

For the purpose of this report, DNT is defined as the adverse effects of substances (regulated foreign compounds or xenobiotics) on the nervous system associated with exposure during development. The adverse effects may be expressed at any time during the life span of the exposed individual.

Available Tests Linked to DNT End Points, Processes, and Models

Alternative approaches to DNT testing can be divided into two classes: in vitro models and nonmammalian animal models. In the following section we summarize possible in vitro models for DNT testing and then elaborate in more detail on nonmammalian models for DNT testing, and conclude with a critical assessment of these approaches for DNT testing.

In vitro models for DNT testing. Many neural development processes are understood...
at a cellular and molecular level, and can partly be modelled in vitro. Cell culture techniques have been developed to address key biochemical and functional features of developmental neural biology such as cell migration, formation of neuronal networks, synaptogenesis, and neuron–glia interaction. Among others, these processes may be specifically targeted by developmental neurotoxicants in vitro. Therefore, the rationale for the use of in vitro models for DNT testing is based on a clear understanding of the mechanistic processes underlying normal nervous system development. However, each in vitro model has its own specific advantages and disadvantages (Tiffany-Castiglioni 2004) and represents different grades of complexity and allows predictions for humans to different degrees (Table 1).

In the following sections, we outline the potential uses of the less well-established but promising murine and human embryonic stem cells (ESC) and human neuronal stem cells (NSC) for DNT testing. For a brief discussion on the predictive capacity and inherent limitations of established in vitro models with potential use for DNT testing, see Tables 1 and 2 and the literature therein.

**Rodent and human stem cells.** Presently, the mouse embryonic stem cell test (EST) is the only system based on a mammalian cell line, which was successfully validated as an alternative for in vivo embryotoxicity testing (Genschow et al. 2002). Committing mouse ESCs into neuroectodermal fate or directing these cells to more advanced stages of neuronal development may extend the mouse EST to capture also DNT end points (Haren et al. 2005). Similarly, human ESCs can be directed into all three major central nervous system (CNS) cell types, and the sensitivity of these cell types to compounds may be assessed (Zhang et al. 2001). However, human ES cell culture techniques still require optimization for DNT testing.

Another stem cell model for DNT testing might be somatic neural stem cell (sNSC) cultures. These cultures are characterized by their capacity to self-renew and to differentiate into neurons, astrocytes, and oligodendrocytes. These three cell types can interact with each other in two- and three-dimensional (neurosphere-like structures) cultures. This may provide the ability to assess the sensitivity of early and advanced human neural development to compounds by various means such as cell proliferation, cell migration, cell-type specific mRNA/protein expression, and electrophysiologic responses. Although sNSCs are still a fairly immature model, recent work on normal human neural progenitor cells and a

### Table 1. General characteristics of potential in vitro models for DNT testing.

| Type of culture               | Relevance for DNT                                                                 | Main advantages                                                                                                            | Main limitations                                                                                         |
|------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
| Organotypic cultures         | • Derived from undifferentiated embryonic brain or spinal cord tissue (e.g., slices, explants) (1995a, 1995b, 1997, 2000); Sales et al. (2004); Seeds and Vater (1971); Zurich et al. (2002, 2004). | • Presence of in vivo–like three-dimensional anatomic and functional organization such as tissue-specific cytoarchitecture, neuronal connectivity, electrophysiological activity, complex glial–neuronal interactions | • Low throughput                                                                                                                                 |
|                              | • Develop into mature and interactive neuronal–glial tissue-like structure        | • Presence of neuronal cell types corresponding to the original tissue                                                                 | • Anatomic organization present in the original tissue is lost                                                                 |• Most neurons are postmitotic at culture initiation                                                                                                                                 |
|                              | • Used to study the mechanisms of morphologic and physiologic cell maturation that could be affected by toxicants | • Presence of all glial cell types, i.e., astrocytes, oligodendrocytes, microglia, Glial cell proliferation and maturation, synaptogenesis, and myelination recapitulate in vivo development | • Not suitable for studies at the single-cell level                                                                                                                                 |
| Re-aggregating brain cell culture | • Derived from dissociated embryonic brain cells that re-aggregate spontaneously under continuous gyratory agitation (2005). | • Formation of natural extracellular matrix                                                                                     | • Variability between individual aggregates with respect to size, proportion of neurons versus glial cells, and electrical activity |
|                              | • Reproduce 3D complexity, exhibiting a developmental pattern both morphologically and functionally similar to the original brain tissue in vivo | • Mature cultures exhibit spontaneous and evoked electrical activity                                                                 |                                                                                                                                                                   |
|                              |                                                                                  | • Possibility to study microglial cell activation and astroglial reactivity as early markers of neurotoxicity                  |                                                                                                                                                                   |
|                              |                                                                                  | • Cultured in chemically defined medium                                                                                       |                                                                                                                                                                   |
|                              |                                                                                  | • The model is robust and provides large amount of material for multidisciplinary and multiparametric assays               |                                                                                                                                                                   |
| Primary dissociated culture  | • Used for mechanistic studies and characterization of endogenous factors that are crucial for the normal differentiation and function of the developing nervous system (1996); Overstreet et al. (1997); Pinzor-Duarte et al. (2004); Stoppini et al. (1991); Strasser and Fischer (1995); Zimmer et al. (2000). | • Easy access to single-cell toxicity assay assessment                                                                     | • Histotypic tissue organization is lost                                                                                                                         |
|                              |                                                                                  | • Most of the neurodevelopmental features are preserved (cell death, glia progenitor proliferation, cell migration, synthesis of their receptors or formation of neuronal connections) | • Isolated neurons are postmitotic                                                                                                                                 |
|                              |                                                                                  | • Possibility to use pure culture of each cell type or as mixed neuronal–glial culture to study their interaction | • Variability between neuronal–glial ratio                                                                                                                         |
| Immortalized human and rodent cell lines | • Derived from tumors or transformed cells (neuroblastomas, gliomas, and schwannoma cell lines) (2005). | • Availability of human tissue                                                                                                 | • Because they are transformed cells, differentiation process may not be comparable to “normal” cells, and their ultimate phenotype are often different from primary neurons |
|                              | • Suitable to study the mechanisms of cell differentiation because under the appropriate culture conditions (e.g., exposure to growth factors) the cells differentiate into non-dividing neuroblast-like cells, characterized by neurite outgrowth (2006). | • Differentiated neuronlike cells express electrical activity, synthesis of various neurotransmitters, and expression of associated receptors and ion channels. | • Often neurites are not representative of either axons or dendrites and do not form functional synapses                                                                 |
|                              |                                                                                  | • Provide homogeneous cell populations in large quantities in a very reproducible manner                                      | • Usually only one cell type is present, cell–cell interaction is missing                                                                                                                                 |
|                              |                                                                                  | • Because they are transformed cells, differentiation process may not be comparable to “normal” cells, and their ultimate phenotype are often different from primary neurons | • Genetic instability with increased number of passages                                                                                                               |
nonimmortalized human cord blood–derived NSC line points to its potential for DNT testing (Buzanska et al. 2005; Fritsche et al. 2005).

**Alternative nonmammalian models for DNT testing.** Current *in vivo* test methods for detecting neurotoxicity and DNT are based on a number of end points including behavioral tests that are considered by regulators as crucial for neurotoxicity risk assessment. *In vitro* models cannot recapitulate such complex end points. However, using nonmammalian species in alternative test strategies may help address some of these behavioral end points. Among nonmammalian model organisms, zebrafish and *C. elegans* are particularly suited to address neurotoxic and DNT end points. The ease of obtaining high numbers of progeny, the availability of neuronal tissue specific in *in vivo* reporter strains and the inherent transparency of the embryos make these two model organisms amenable to high-throughput screening (HTS) (Peterson et al. 2000; Wittebrodt et al. 2002). Moreover, the basic understanding of gene function and physiology combined with well-characterized stereotypic behaviors provides the possibility of using zebrafish and *C. elegans* for neurotoxicity risk assessment based on behavioral end points (Orger et al. 2004). Additional nonmammalian species include the sea urchin (Buznikov et al. 2001; Cameron and Davidson 1991) and *Drosophila* (Grueber and Jan 2004; Jones et al. 2006); both are potentially useful systems, but details are not included here due to space limitations.

**Medaka and zebrafish as potential models for DNT.** Assays based on medaka and zebrafish measure general DNT end points such as cell proliferation, neuronal precursor differentiation, and maturation. The existing techniques allow the assessment of specific neuronal migration, axonal and dendritic outgrowths, pruning, synaptogenesis, development of neuronal circuits, and their ultimate function—behavior (Tables 2 and 3).

Neuronal precursor proliferation and molecular differentiation can be assayed in *in vitro* and *in vivo*. In *in vitro*, marker gene expression can be used to analyze the specification of major neuronal and glial cell types (neurons, oligodendrocytes, astrocytes, microglia) and neuronal subtypes (e.g., GABAergic neurons, glutaminergic neurons) (Brosamle and Halpern 2002). More recently, transgenic techniques allow the analysis of these marker genes in live animals by fluorescent protein expression (Higashijima et al. 2000; Park et al. 2000; Table 3).

Similarly, neuronal migration and morphologic differentiation can also be analyzed in *in vitro* and *in vivo*. In *in vitro*, a combination of marker gene expression and cell position can be used to assess neuronal migration. However, this is an indirect measure because incorrect patterning and morphogenesis of other tissues may affect neuronal migration and, thus, correct positioning. These caveats may, in part, be overcome by *in vivo* analyses of neuronal migration. Using transgenic techniques to fluorescently mark migrating cells, *in vivo* analyses of migration also offer the possibility to assess other cell dynamics besides correct cell positioning (Gilmour et al. 2002). Although more laborious, this allows motility and chemotaxis defects during neuronal migration to be distinguished, and may resolve

### Table 2. Overview of promising in vitro alternative models and their characteristics.

| In vitro models/processes | Human stem cell/precursor cells | Rodent stem cells | Immortalized human cell lines | Immortalized rodent cell lines | Brain aggregates | Primary dissociated cultures | Organotypic cultures |
|--------------------------|---------------------------------|------------------|-----------------------------|-------------------------------|----------------|---------------------------|----------------------|
| Cell proliferation       | Li et al. 2005                   | Milosevic et al. 2005 | Shastry et al. 2001         | Margioris et al. 1995         | (glial only) Honegger and Richelson 1979 | (glial only) Kinsner et al. 2005 | —                    |
| Precursor cell differentiation | Carpenter et al. 2001          | Takahashi et al. 1999 | Shastry et al. 2001         | Greene 1978                 | (glial only) | +                          | Pinedo-Duarte et al. 2004 |
| Glial reactivity         | Turkla et al. 1995              | Martinez-Contreras et al. 2002 | —                          | Seidman et al. 1997          | + Monnt-Tsudhi et al. 1995a, 1995b; Zurich et al. 2002 | + Bal-Price and Brown 2001 | + Neumann 1996 |
| Glial maturation (myelination) | Winderm et al. 2004           | Brustle et al. 1999 | Liu et al. 2000 Peden et al. 1990 | Garcia et al. 2001           | Honegger and Matthieu 1985 | Demersen et al. 1996 | + Ghoumari et al. 2005 |
| Migration                | Imitola et al. 2004             | Imitola et al. 2004 | —                          | —                            | Levitt et al. 1976 | + Sass et al. 2001 | + Heck et al. 2006 |
| Axon/dendritic outgrowth | Harper et al. 2005              | Yoon et al. 2005 | Abdulla et al. 1995         | Parran et al. 2001           | Braissant et al. 2002 | Howard et al. 2005 | + Hechler et al. 2006 |
| Apoptosis                | Li et al. 2006                   | Milosevic et al. 2005 | Ba et al. 2003              | Pittman et al. 1993          | Monnt-Tsudhi 1998 | + Desi et al. 1995 | + Chaliysova et al. 2006 |
| Synapse formation        | Cummings et al. 2005            | Copi et al. 2005 | —                          | —                            | Seeds and Vater 1971; Monnt-Tsudhi et al. 1995b | + Yamamoto et al. 2005 | + Chen et al. 2005 |
| Synapse pruning          | —                               | —                | —                          | —                            | Goldin et al. 2001 | Overstreet et al. 1997 | + Zimmer et al. 2000 |
| Neurotransmitter receptor profiles | Zhang et al. 2001; Carpenter et al. 2001 | Ma et al. 2004 | Lambert and Nahorski 1990 | Whiting et al. 1987 | Honegger and Richelson 1979 | Sah and Matsumoto 1987 | + |
| Neuronal connectivity    | Benninger et al. 2003           | Toda et al. 2000 | —                          | —                            | — van Pelt et al. 2005 | —                        | —                    |

*Grading of technical feasibility and accessibility of in vitro alternative models available: ###, complex; ##, less complex; #, simple. Grading of availability and extend of literature related to the processes for a specific alternative model: ++, currently available; +, potential; —, not yet available. Some of these systems are high-throughput, whereas others have the potential to be developed.
secondary defects better than static in vitro analyses (Table 3).

Morphologic differentiation of neurons involves dynamic processes. Neurons extend axons and dendrites, establish and discontinue synaptic contacts, and mature into a neuronal circuit. In vitro, neuronal membrane–specific antibodies and, in vivo, membrane-tethered fluorescent proteins can be used to analyze these processes (Higashijima et al. 2000; Park et al. 2000).

Behavior is the ultimate effect of neuronal development. Certain behavioral responses of fish can be analyzed reasonably robustly, because some of the underlying neuronal circuits have been characterized (Baier 2000). Such behaviors may be classically analyzed by responses to certain stimuli or, more recently, molecularly by stimulus-induced gene expression.

Limitations of alternative models for DNT testing. The alternative approaches to DNT testing discussed above and in Tables 1–3 are potential models for DNT testing. To our knowledge none of these models have been used specifically for DNT testing up to this date. However, we believe that these models merit intensive consideration in drafting DNT testing strategies if one is aware of their limitations and caveats.

One concern is the predictive capacity of alternative models. How does one interpret an “effect” seen in an in vitro or nonmammalian model? Provided the molecular basis leading to the end point in question reflects in vivo development, an “effect” detected in an alternative model is a good indication that a similar effect may be expected in humans after chemical exposure. This is less of a concern when using nonmammalian models for DNT testing because mechanisms of neural development are highly conserved among distant species. On the other hand, interspecies differences may cause false positives or false negatives when screening compounds for adverse effects on humans, with nonmammalian models. The use of human cell systems will circumvent this problem. Therefore, a combination of nonmammalian and human cell–based models may maximize the predictiveness of alternative models.

Pharmacokinetics need to be considered when interpreting results from in vitro models. Because in vitro systems do not reflect in vivo absorption, distribution, metabolism, and excretion (ADME) of test compounds, results need to be interpreted with caution. This also holds true for nonmammalian in vivo models. These systems display ADME phenomena, but these phenomena do not necessarily reflect the human situation.

An additional, more practical concern is the amenability to automation and HTS. Depending on the model, the feasibility of HTS varies, and Tables 2 and 3 show to what extent we believe the different models were suited for such an approach.

As indicated above, some of the discussed caveats may be partly overcome by combining different alternative models in an intelligent testing strategy. Models with a well-characterized mechanism that reflects the in vivo situation, possibly based on human cell lines, in combination with nonmammalian models may more robustly detect compounds with adverse effects on humans. In addition, nonmammalian models may mimic human pharmacokinetics to a certain degree and offer the possibility to assess basic neuronal network functions such as simple behavior, although more complex behaviors relying on neocortical structures unique to mammals will escape detection. In this light, an intelligent combination of DNT tests may help refine the in vivo animal histology and behavioral testing battery used in the U.S. EPA and OECD guidelines.

### Systems Interaction and other Considerations

**Interaction/interplay between endocrine and immune function.** Chemicals may interfere with or mimic the effects of endogenous hormones and signaling chemicals of the endocrine system. A well-known example is the interference of chemicals with the thyroid system (Colborn 2004; Damstra 2002). There are international efforts to validate or standardize screens or assays for detecting test chemicals with potential endocrine-disrupting effects. Among the chemicals on the market today, few if any have been systematically tested for such effects for regulatory purposes. These tests should be integrated in an overall hazard and risk assessment strategy for DNT.

**Importance of blood–brain barrier and choroid plexus in DNT.** Alterations in both BBB and choroid plexus (CP) have been implicated in neurodevelopmental disorders. The integrity of the BBB and the CP, both structurally and functionally, is essential for brain chemical stability. In vitro BBB and CP models (Prieto et al. 2004; Reichel et al. 2003) are available and can be used for evaluating the DNT potential of chemicals. The BBB is a special capillary bed that separates the blood from the CNS parenchyma. The CP produces the cerebrospinal fluid (CSF) and is involved in the most basic aspects of neural function including: maintaining the extracellular milieu of the brain by actively modulating chemical exchange between the CSF and the brain parenchyma, surveying the chemical and immunologic status of the brain, detoxifying the brain, secreting a nutritive mixture of polypeptides, and participating in repair processes after trauma (Emerich et al. 2005). Retrospective studies diagnosing human fetal anomalies of the CNS by ultrasonography and by fetal magnetic resonance imaging, combined with follow-up studies after birth, have revealed CP pathology in 9% of children with suboptimal neurodevelopmental (Leitner et al. 2004).

**Metabolism-mediated DNT effects.** Biokinetic processes—such as absorption, distribution, biotransformation, and excretion—determine the relationship between the exposure pattern and the internal concentration...
time course. In addition to barrier effects, biotransformation may play a crucial role in DNT hazards. Biotransformation or metabolism is the process by which a substance in the body is chemically transformed to a metabolite or a variety of metabolites. Biotransformation is usually divided into two main phases, phase 1 and phase 2, the former of which is usually oxidative (e.g., hydrolysis, although reductive metabolism can occur) and predominantly catalysed by the many isoforms of the cytochrome P450 supergene family. Phase 2 is catalysed by a variety of enzymes that conjugate the oxidized moiety with highly polar molecules, such as glucose, sulphate, methionine, cysteine, or glutathione. The biokinetics of a compound, including its metabolism, can greatly influence its toxicologic properties. One of the most frequently cited limitations of nonhuman-based in vitro and in vivo assays is the qualitative and quantitative differences in the biotransformation of test chemicals, in comparison with human biotransformation (Coecke et al. 2006). The effect of human chemical biotransformation needs to be taken into account in both in vivo and in vitro assays. In the case of DNT, existing data indicate a role of biotransformation for DNT (Parmar et al. 2003). Estrogen formed in the brain and from other estrogen-synthesizing tissues is catalyzed by cytochrome P450 aromatase isoforms. Estrogen regulates neuronal, proliferation, survival, morphology, synaptogenesis, and differentiated functions in many various regions of the adult brain. Thus, inhibition or induction of these cytochrome P450 isoforms may cause alterations in these processes.

**Validation and Testing Strategies**

To investigate chemicals that have the potential to cause DNT, we have illustrated the availability of in vitro and in vivo test methods. Both in vitro and nonmammalian test systems (particularly when used in combination) offer the possibility of providing an early screen for a large number of chemicals, and could be useful in characterizing the mechanism of action or the developmental processes that are particularly affected by the test chemical. In vitro assays may not always reflect the in vivo animal results because of species differences, absence of kinetic considerations, or a complex interplay between a diverse range of mechanisms and processes affected by the chemicals, including for example, the interaction/interplay between endocrine and immune function. Therefore, a battery of in vitro and in vivo assays seems at present the most appropriate way of providing the added value of the alternative approaches.

Any potential alternative test system must be validated and standardized before the information generated can be used for hazard identification and for risk assessment. Because adequate reference methods that reliably predict health effects are lacking and the in vivo approaches are complex, the assessment of the in vitro methods will not be a straightforward task. A significant problem with the existing in vivo methods for the identification of developmental neurotoxicants is the lack of explicit guidance on how to quantitate the risks of DNT (either for low observed effect level (LOEL) or no observed effect level (NOEL), or for benchmarks). Moreover, it is difficult to interpret the methods in terms of their predictive value for human health. Figure 1 illustrates a strategy for toxicologic evaluation in the context of DNT testing, including the possible contribution of both in vitro and nonmammalian testing.

**Tier 1.** Tier 1 incorporates existing knowledge including a) any animal studies, b) in vitro studies (cell and tissue cultures methods, conventional and novel end points such as “omics”), c) exposure information, d) epidemiologic information, e) intended use, and f) chemical structure and any relevant physicochemical data. The first step is a critical evaluation of the quality of existing information. If sufficient information is available at Tier 1, a decision can be made if there is a concern about DNT. If there are data gaps, new in vivo data should be generated and complemented by in vitro testing to enable a decision to be made regarding DNT potential. In cases where absolutely no chemical information exists, a base set of data should be generated [see OECD recommendations on Screening Information Data sets (SIDs) (International Programme on Chemical Safety 1996)]. Both in vitro and nonmammalian test systems could be applied for initial screening to permit an approximate DNT assessment.

**Tier 2.** In cases where data are available, a decision can be made whether there is a DNT concern (high or low priority). In cases where there is evidence for pre- and/or postnatal exposure in humans (e.g., detection of a chemical in breast milk), priority should be high. If information collected in Tier 1 is relevant to DNT, priority should be given for DNT testing. The evaluation of the exposure scenario will aid the decision as to whether the compounds are high or low priority for DNT testing, or if no further testing is required and a regulatory action can be taken. Well-conducted studies that indicate no current concern should move chemicals to the low-priority list for DNT testing. These compounds should be evaluated when new information becomes available from animal and alternative test methods. In cases where available data support a high concern for DNT, Tier 3 testing should be carried out.

**Tier 3.** Based on the nature of the available data and regulatory requirements, this stage could include very specific tests, or it may involve the use of higher-order in vitro or nonmammalian alternative tests, or in vivo mammalian testing.

However, in vitro or nonmammalian alternative approaches may become important for this stage when the number of chemicals with no available data is very high. This is currently the case in different international regulatory environments. The drivers in Europe, for the research of alternative methods to replace conventional animal tests for toxicologic hazard assessments, are generally related to the chemical and cosmetic regulations (Escues and Zuang 2005; European Commission 2003). Also, with the High Production Volume Challenge Program in the United States (U.S. EPA 1998), a call for the replacement of animal experiments within a short-time frame is being launched. Therefore, Tier 3 in the in vitro alternative predictive test batteries strategy, based on end points, mechanisms, and processes relevant to DNT for chemical-induced neurotoxicity, might be of great added value and become increasingly more important for regulatory decision making. Tests may include the use of in silico tools in combination with one or more of the proposed alternative test systems targeting specific mechanistic or functional markers of developmental neurotoxicant-induced alterations. Such tests may be integrated genomic, proteomic, and other “omic” analyses and a variety of biochemical, morphologic, biotechnologic, or electrophysiologic profiling methods. Such a tiered testing scheme will allow more data to be generated in Tier 3 for those compounds where there is a concern for DNT, to allow regulatory decision making. Such tiered approaches that integrate batteries of in vitro alternative tests are currently proposed for ecotoxicologic risk assessment (Jeram et al. 2005).

**Conclusion**

There is societal concern that the increasing prevalence of childhood behavioral diseases is
related to augmented exposures to xenobiotics. Currently, there are thousands of chemicals that have not been evaluated for their potential to cause DNT. DNT tests used for regulatory decisions should predict and identify DNT hazards. Despite the empirical usefulness of in vivo animal tests, these tests consume a high number of animals, are labor intensive, complex in experimental design, not always sensitive enough, and often do not provide information that facilitates a detailed understanding of potential mechanisms of toxicity. Furthermore, in vivo mammalian tests are unsuitable for screening large numbers of agents, and their predictive capacity for the human situation remains unclear. Today, it would be too ambitious to pretend that an alternative DNT strategy would focus on replacement of the in vivo DNT test methods. A first step would be to refine the current in vivo strategies by integrating information derived from in vitro and nonmammalian alternative test strategies. In vitro DNT tests designed to identify chemicals with the potential to cause DNT would eliminate the number of chemicals reliant on DNT data solely from in vivo mammalian DNT tests, and therefore reduce animal testing. At the same time, primary neuronal cell cultures (organotypic, re-aggregating, or dissociated) are prepared from freshly isolated brain tissue, thus still consuming animals, although one animal provides material for many individual experiments. Because at present in vivo–based DNT testing cannot be replaced by in vitro approaches, incorporation of in vitro testing as a part of an intelligent testing strategy could at least refine and eventually reduce animal usage.

Combining in vivo data sets with in vitro approaches in intelligent test strategies is increasingly important for regulatory decision making. In addition, these approaches will also be the most efficient way to decrease costs and the amount of time required for testing. In this report we focused on the several processes involved in brain development and identified in vitro and nonmammalian tests that may allow the generation of data sets that can help identify DNT hazards. Although all the test systems described were not developed for regulatory purposes at this stage, if they prove useful, we hope that this report will encourage their further development to render them amenable to high-throughput approaches. In this context we consider a test system “useful” if it models a certain in vivo process and distinguishes known chemicals that interfere or do not interfere with this process. Thus, this report proposes an approach that would refine and potentially reduce the number of animal tests that need to be performed. Most important, test strategies/batteries are needed that can evaluate mechanisms responsible for DNT.

**Recommendations**

1. **Focus on experimental designs relevant for regulatory methods.**
   - Catalog in vitro systems available in the research community (including academia and government), and explore how they can be developed for regulatory use.
   - A reference list of potential DNT chemicals should be established that incorporates all available data. Benchmarking against experiences gained in other large international efforts (e.g., ReProTect; Hareng et al. 2005) is suggested.
   - Proper experimental design of in vitro (and in vivo) DNT tests must have positive and negative controls. Therefore, there is an urgent need to generate high-quality data on chemicals with DNT potentials.

2. **Toward development of an integrated tiered approach.**
   - Further refine the tiered approach based on the integration of physico-chemical data, and other data sets available for the in vivo/in vitro toxicologic effects; exposure use patterns, toxicokinetic, immunotoxic, reproductive toxicity, endocrine disruption and available developmental toxicity data sets should be explored.
   - Evaluate the introduction of an iterative tiered/battery approach for DNT testing that combines both in vivo and in vitro data sets.
   - Increase knowledge of critical neurodevelopmental processes enabling comparisons of alternative DNT models.

3. **A strategy should be developed to evaluate reference chemicals in multiple models to identify those models to be considered for inclusion in an intelligent testing strategy.**

4. **Evaluate, by literature searches, which end points and mechanisms are linked to adverse effects on the developing human nervous system.**

5. **Catalog and correlate human neurodevelopmental landmarks to findings/end points from animal and in vitro studies.**

6. **Alternative approaches using contemporary human and rodent cell and tissue cultures and alternative species might be the way forward to achieve a greater understanding of the importance of considering species differences when evaluating the DNT hazards of xenobiotics.**

7. **Considerations should be given to metabolism-mediated toxic effects (inclusion of metabolic competence).**

8. **With the recognition of the lack of knowledge in developmental biology and developmental neurotoxicology, we encourage exploration of the most fundamental research issues.**

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