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, myelinization, 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

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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 vivo*. 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 (Hareng 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[a]	• Derived from undifferentiated embryonic brain or spinal cord tissue (e.g., slices, explants) • Develop into mature and interactive neuronal–glial tissue-like structure • Used to study the mechanisms of morphologic and physiologic cell maturation that could be affected by toxicants	• Presence of *in vivo*–like three-dimensional anatomic and functional organization such as tissue-specific cytoarchitecture, neuronal connectivity, electrophysiologic activity, complex glial–neuronal interactions	• Low throughput • Limited period of culture • Possible necrosis in the tissue center due to the limited oxygen and nutrients supply
Re-aggregating brain cell culture[b]	• Derived from dissociated embryonic brain cells that re-aggregate spontaneously under continuous gyratory agitation • Reproduce 3D complexity, exhibiting a developmental pattern both morphologically and functionally similar to the original brain tissue *in vivo*	• Presence of neuronal cell types corresponding to the original tissue • Presence of all glial cell types, i.e., astrocytes, oligodendrocytes, microglia • Glial cell proliferation and maturation, synaptogenesis, and myelination recapitulate *in vivo* development • Formation of natural extracellular matrix • 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	• Anatomic organization present in the original tissue is lost • Most neurons are postmitotic at culture initiation • Not suitable for studies at the single-cell level • Variability between individual aggregates with respect to size, proportion of neurons versus glial cells, and electrical activity
Primary dissociated culture[c]	• Used for mechanistic studies and characterization of endogenous factors that are crucial for the normal differentiation and function of the developing nervous system	• Easy access to single-cell toxicity assay assessment • Most of the neurodevelopmental features are preserved (cell death, glia progenitor proliferation, cell migration, synthesis of transmitters, and expression of their receptors or formation of neuronal connections) • Possibility to use pure culture of each cell type or as mixed neuronal–glial culture to study their interaction • Easy to obtain and maintain	• Histotypic tissue organization is lost • Isolated neurons are postmitotic • Variability between neuronal–glial ratio
Immortalized human and rodent cell lines[d]	• Derived from tumors or transformed cells (neuroblastomas, gliomas, and schwannoma cell lines) • Suitable to study the mechanisms of cell differentiation because under the appropriate culture conditions (e.g., exposure to growth factors) the cells differentiate into nondividing neuronlike cells, characterized by neurite outgrowth	• Availability of human tissue • Differentiated neuronlike cells express electrical activity, synthesis of various neurotransmitters, and expression of associated receptors and ion channels. • Provide homogeneous cell populations in large quantities in a very reproducible manner	• Because they are transformed cells, differentiation process may not be comparable to "normal" cells, and their ultimate phenotype are often different from primary neurons • Often neurites are not representative of either axons or dendrites and do not form functional synapses • Usually only one cell type is present, cell–cell interaction is missing • Genetic instability with increased number of passage

[a]Data on organotypic cultures from Braun et al. (2006); Chalisova et al. (2006); Chen et al. (2005); Ghoumari et al. (2005); Hechler et al. (2006); Heck et al. (2006); Neumann et al. (1996); Overstreet et al. (1997); Pinzon-Duarte et al. (2004); Stoppini et al. (1991); Strasser and Fischer (1995); Zimmer et al. (2000). [b]Data on re-aggregating brain cell culture from Braissant et al. (2002); Braun et al. (2006); Eskes et al. (1999, 2002); Harry et al. (1998); Honegger (1985); Honegger and Monnet-Tschudi (2001); Honegger and Richelson (1979); Monnet-Tschudi et al. (1995a, 1995b, 1997, 2000); Sales et al. (2004); Seeds and Vater (1971); Zurich et al. (2002, 2004). [c]Data on primary dissociated culture from Bal-Price and Brown (2001); Demerens et al. (1996); Goldin et al. (2001); Howard et al. (2005); Krause et al. (2006); Lein et al. (1995); Sah and Matsumoto (1987); Sass et al. (2001); Veronesi (1992); van Pelt et al. (2005); Yamamoto et al. (2005). [d]Data on immortalized human and rodent cell lines from Abdulla et al. (1995); Greene (1978); Hong et al. (2003); Pahlaman et al. (1990); Parran et al. (2001); Sachana et al. (2001).

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 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; Wittbrodt 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 *Drosphila* (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 vitro* and *in vivo*. *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 vitro* and *in vivo*. *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 offers 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[a] and their characteristics.

In vitro models/ processes[b]	Human stem cell/ precursor cells ###	Rodent stem cells ###	Immortalized human cell lines neuronal/ nonneuronal #	Immortalized rodent cell lines neuronal/ nonneuronal #	Brain aggregates ###	Primary dissociated cultures ##	Organotypic cultures ##
Cell proliferation	++ Li et al. 2005 Zhang et al. 2001	++ 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) Guentert-Lauber and Honegger 1985	—	+ Pinzon-Duarte et al. 2004
Glial reactivity	+ Turka et al. 1995	+ Martinez-Contreras et al. 2002	—	++ Seidman et al. 1997	++ Monnet-Tschudi et al. 1995a, 1995b; Zurich et al. 2002	++ Bal-Price and Brown 2001	+ Neumann et al. 1996
Glial maturation (myelination)	+ Windrem et al. 2004	+ Brüstle et al. 1999	++ Liu et al. 2000 Peden et al. 1990	++ Garcia et al. 2001	++ Honegger and Matthieu 1985	++ Demerens 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. 2004	++ 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. 2005	++ Milosevic et al. 2005	++ Ba et al. 2003	++ Pittman et al. 1993	++ Monnet-Tschudi 1998	++ Dessi et al. 1995	++ Chalisova et al. 2006
Synapse formation	+ Cummings et al. 2005	++ Copi et al. 2005	—	—	++ Seeds and Vater 1971; Monnet-Tschudi et al. 1995b	++ Yamamoto et al. 2005	++ Chen et al. 2005
Synapse pruning			—	—	—	++ Goldin et al. 2001	+ Overstreet et al. 1997
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	++ Zimmer et al. 2000
Neuronal connectivity	+ Benninger et al. 2003	++ Toda et al. 2000	—	—	—	++ van Pelt et al. 2005	—

[a]Grading of technical feasibility and accessibility of *in vitro* alternative models available: ###, complex; ##, less complex; #, simple. [b]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.

Table 3. Overview of promising nonmammalian alternative models[a] and their characteristics.

Nonmammalian models/ processes[b]	Zebrafish ##	Medaka ##	*C. elegans* #
Cell proliferation	++ Wullimann and Knipp 2000	++ Candal et al. 2005	++ van den Heuvel 2005
Precursor cell differentiation	++ Bertrand et al. 2002	++ Hirose et al. 2004	++ Chisholm and Jin 2005
Glial reactivity	—	—	—
Glial maturation (myelination)	+ Brosamle and Halpern 2002	—	—
Migration	++ Gilmour et al. 2002	++ Hirose et al. 2004	++ Hatten 2002
Axon/dendritic outgrowth	++ Beattie et al. 2002	++ Ishikawa et al. 2004	++ Colamarino and Tessier-Lavinge 1995; Cooper 2002
Apoptosis	++ Cole et al. 2001	++ Stokes et al. 2004	++ Lettre and Hengartner 2006
Synapse formation	++ Hutson and Chien 2002	—	++ Ackley and Jin 2004; Jin 2002
Synapse pruning	++ Hutson and Chien 2002	—	++ Wiggin et al. 2005
Neurotransmitter receptor profiles	++ Higashijima et al. 2004	++ Hamm et al. 2001	++ Komuniecki et al. 2004
Neuronal connectivity	++ Godinho et al. 2005	—	++ Volovitch et al. 1993

[a]Grading of technical feasibility and accessibility of *in vitro* nonmammalian models available: ###, complex; ##, less complex; #, simple. [b]Grading of availability and extent of literature related to the processes for a specific alternative model: ++, currently available; +, potentially available; —, not yet available. Some of these systems are amenable for high-throughput screening.

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 barriers, 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 vivo* and *in vitro* 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*) epidemiology 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 chemicals and cosmetic regulations (Eskes 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 use 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

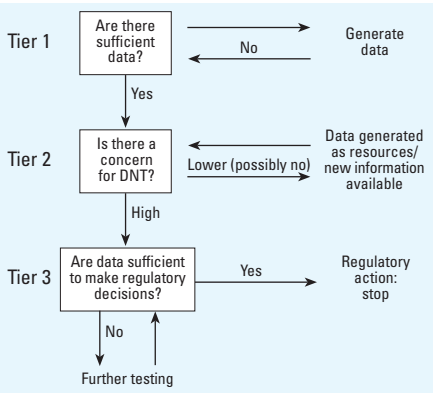

Figure 1. Scheme of the different steps in identifying DNT hazards. Two different levels were identified.

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

- Focus on experimental designs relevant for regulatory methods.
  1. Catalog *in vitro* systems available in the research community (including academia and government), and explore how they can be developed for regulatory use.
  2. 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.
  3. 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.
- Toward development of an integrated tiered approach.
  1. 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, immunotoxicity, reproductive toxicity, endocrine disruption and available developmental toxicity data sets should be explored.
  2. Evaluate the introduction of an iterative tiered/battery approach for DNT testing that combines both *in vitro* and *in vivo* data sets.
- Increase knowledge of critical neurodevelopmental processes enabling comparisons of alternative DNT models.
  1. 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.
  2. Evaluate, by literature searches, which end points and mechanisms are linked to adverse effects on the developing human nervous system.
  3. Catalog and correlate human developmental landmarks to findings/end points from animal and *in vitro* studies.
  4. 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. Until interspecies differences for DNT are better understood, use of both human and rodent cell and tissue cultures and alternative species should continue.
  5. Considerations should be given to metabolism-mediated toxic effects (inclusion of metabolic competence).
  6. With the recognition of the lack of knowledge in developmental biology and developmental neurotoxicology, we encourage exploration of the most fundamental research issues.
  7. A long-term goal should be to evaluate the use of "omics" in DNT assessment.

References

Abdulla EM, Calaminici M, Campbell IC. 1995. Comparison of neurite outgrowth with neurofilament protein subunit levels in neuroblastoma cells following mercuric oxide exposure. Clin Exp Pharmacol Physiol 22:362–363.

Ackley BD, Jin Y. 2004. Genetic analysis of synaptic target recognition and assembly. Trends Neurosci 27:540–547.

Ba F, Pang PK, Benishin CG. 2003. The establishment of a reliable cytotoxic system with SK-N-SH neuroblastoma cell culture. J Neurosci Meth 123:11–22.

Baier H. 2000. Zebrafish on the move: towards a behavior-genetic analysis of vertebrate vision. Curr Opin Neurobiol 10:451–455.

Bal-Price A, Brown GC. 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21:6480–6491.

Beattie CE, Granato M, Kuwada JY. 2002. Cellular, genetic and molecular mechanisms of axonal guidance in the zebrafish. Results Probl Cell Differ 40:252–269.

Benninger F, Beck H, Wernig M, Tucker KL, Brustle O, Scheffler B. 2003. Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. J Neurosci 23:7075–7083.

Bertrand N, Castro DS, Guillemot F. 2002. Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517–530.

Braissant O, Henry H, Villard A-M, Zurich M-G, Loup M, Eilers B, et al. 2002. Ammonium-induced impairment of axonal growth is prevented through glial creatine. J Neurosci 22:9810–9820.

Braun H, Buhnemann C, Neumann J, Reymann KG. 2006. Preparation of a tissue-like cortical primary culture from embryonic rats using Matrigel and serum free Start V Medium. J Neurosci Methods 15:32–38.

Brosamle C, Halpern ME. 2002. Characterization of myelination in the developing zebrafish. Glia 39:47–57.

Brüstle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD. 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285:754–756.

Buzanska L, Habich A, Jurga M, Sypecka J, Domanska-Janik K. 2005. Human cord blood-derived neural stem cell line-possible implementation in studying neurotoxicity. Toxicol *In Vitro* 19:991–999.

Buznikov GA, Lambert HW, Lauder JM. 2001. Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis. Cell Tissue Res 305:177–186.

Cameron RA, Davidson EH. 1991. Cell type specification during sea urchin development. Trends Genet 7(7):212–218.

Candal E, Anadon R, Bourrat F, Rodriguez-Moldes I. 2005. Cell proliferation in the developing and adult hindbrain and midbrain of trout and medaka (teleosts): a segmental approach. Brain Res Dev Brain Res 160:157–175.

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 172:383–397.

Chalisova NI, Penniyainen VA, Komashnya AV, Nozdrachev AD. 2006. Stimulation of cell proliferation and apoptosis in the presence of amino acids in organotypic culture of tissues of different degree of maturity. Dokl Biol Sci 406:7–10.

Chen S, Hirata K, Ren Y, Sugimori M, Llinas R, Hillman DE. 2005. Robust axonal sprouting and synaptogenesis in organotypic slice cultures of rat cerebellum exposed to increased potassium chloride. Brain Res 1057:88–97.

Chisholm AD, Jin Y. 2005. Neuronal differentiation in *C. elegans*. Curr Opin Cell Biol 17:682–689.

Coecke S, Ahr H, Blaauboer B, Bremer S, Casati S, Castell J. 2006. Metabolism: a bottleneck in *in vitro* toxicological test development. The report and recommendations of ECVAM workshop 54. ATLA 34:49–84.

Colamarino SA, Tessier-Lavinge M. 1995. The role of the floor plate in axon guidance. Annu Rev Neurosci 18:497–529.

Colborn T. 2004. Neurodevelopment and endocrine disruption. Environ Health Perspect 112:944–949.

Cole LK, Ross LS. 2001. Apoptosis in the developing zebrafish embryo. Dev Biol 240(1):123–142.

Cooper HM. 2002. Axon guidance receptors direct growth cone pathfinding: rivalry at the leading edge. Int J Dev Biol 46:621–631.

Copi A, Jüngling K, Gottmann K. 2005. Activity- and BDNF-induced plasticity of miniature synaptic currents in ES cell-derived neurons integrated in a neocortical network. J Neurophysiol 94:4538–4543.

Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, et al. 2005. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci USA 102:14069–14074.

Damstra T. 2002. Potential effects of certain persistent organic pollutants and endocrine disrupting chemicals on the health of children. J Toxicol Clin Toxicol 40:457–465.

Demerens C, Stankoff B, Logak M, Anglade P, Allinquant B, Couraud F. 1996. Induction of myelination in the central nervous system by electrical activity. Proc Natl Acad Sci USA 93:9887–9892.

Dessi F, Pollard H, Moreau J, Ben-Ari Y, Charriaut-Marlangue C. 1995. Cytosine arabinoside induces apoptosis in cerebellar neurons in culture. J Neurochem 64:1980–1987.

Emerich DF, Skinner SJ, Borlongan CV, Vasconcellos AV, Thanos CG. 2005. The choroid plexus in the rise, fall and repair of the brain. Bioessays 27:262–274.

Eskes C, Honegger P, Jones-Lepp T, Varner K, Matthieu J-M, Monnet-Tschudi F. 1999. Neurotoxicity of dibutyltin in aggregating brain cell cultures. Toxicol *In Vitro* 13:555–560.

Eskes C, Honegger P, Juillerat-Jeanneret L, Monnet-Tschudi F. 2002. Microglial reaction induced by noncytotoxic methylmercury treatments lead to neuroprotection via interaction with astrocytes and IL-6 release. Glia 37:43–52.

Eskes C, Zuang V. 2005. Alternative (non-animal) methods for cosmetics testing: Current status and future prospects, A report prepared in the context of the 7th amendment to the cosmetics directive for establishing the timetable for phasing out animal testing. ATLA 33:1–217.

European Commission. 2003. Proposal Concerning the Registration, Evaluation, Authorisation and Restrictions of Chemicals REACH, COM2003 644 Final 29 Oct. 2003. Brussels:European Commission.

Evangelista de Duffard AM, Duffard R. 1996. Behavioral toxicology, risk assessment, and chlorinated hydrocarbons. Environ Health Perspect 104:353–360.

Fritsche E, Cline JE, Nguyen NH, Scanlan TS, Abel J. 2005. Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. Environ Health Perspect 113:871–876.

Garcia SJ, Seidler FJ, Crumpton TL, Slotkin TA. 2001. Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecular synthesis, adenylyl cyclise signalling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. Brain Res 891:54–68.

Genschow E, Spielmann H, Scholz G, Seiler A, Brown N, Piersma A. 2002. The ECVAM international validation study on *in vitro* embryotoxicity tests: results of the definitive phase and evaluation of prediction models. ATLA 30:151–176.

Ghoumari AM, Baulieu EE, Schumacher M. 2005. Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures. Neuroscience 135:47–58.

Gilmour DT, Maischein HM, Nusslein-Volhard C. 2002. Migration and function of a glial subtype in the vertebrate peripheral nervous system. Neuron 34:577–588.

Godinho L, Mumm JS, Williams PR, Schroeter EH, Koerber A, Park SW, et al. 2005. Targeting of amacrine cell neurites to appropriate synaptic laminae in the developing zebrafish retina. Development 132:5069–5079.

Goldin M, Segal M, Avignone E. 2001. Functional plasticity triggers formation and pruning of dendritic spines in cultured hippocampal networks. J Neurosci 21:186–193.

Greene LA. 1978. Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. J Cell Biol 78:747–755.

Grueber WB, Jan YN. 2004. Dendritic development: lessons from Drosophila and related branches. Curr Opin Neurobiol 14:74–82.

Guentert-Lauber B, Honegger P. 1985. Responsiveness of astrocytes in serum-free aggregate cultures to epidermal growth factor: dependence on the cell cycle and the epidermal growth factor concentration. Dev Neurosci 7:286–295.

Hamm JT, Wilson BW, Hinton DE. 2001. Increasing uptake and bioactivation with development positively modulate diazinon toxicity in early life stage medaka (*Oryzias latipes*). Toxicol Sci 61:304–313.

Hareng L, Pellizzer C, Bremer S, Schwarz M, Hartung T. 2005. The integrated project ReProTect: a novel approach in reproductive toxicity hazard assessment. Reprod Toxicol 20:441–452.

Harper JM, Krishnan C, Darman JS, Deshpande DM, Peck S, Shats I, et al. 2004. Axonal growth of embryonic stem cell-derived motoneurons *in vitro* and in motoneuron-injured adult rats. Proc Natl Acad Sci USA 101:7123–7128.

Harry GJ, Billingsley ML, Bruinink A, Campbell IL, Classen W, Dorman DC. 1998. *In vitro* techniques for the assessment of neurotoxicity. Environ Health Perspect 106:131–158.

Hatten ME. 2002. New directions in neuronal migration. Science 297:1660–1663.

Hechler D, Nitsch R, Hendrix S. 2006. Green-fluorescence-protein-expressing mice as models for the study of axonal growth and regeneration *in vitro*. Brain Res Rev 52:160–169.

Heck N, Kilb W, Reiprich P, Kubota H, Furukawa T, Fukuda A, et al. 2006. GABA-A receptors regulate neocortical neuronal migration *in vitro* and *in vivo*. Cereb Cortex 1:25–35.

Higashijima S, Hotta Y, Okamoto H. 2000. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. J Neurosci 20:206–218.

Higashijima S, Mandel G, Fetcho JR. 2004. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1–18.

Hirose Y, Varga ZM, Kondoh H, Furutani-Seiki M. 2004. Single cell lineage and regionalization of cell populations during Medaka neurulation. Development 131:2553–2563.

Honegger P. 1985. Biochemical differentiation in serum-free aggregating brain cell cultures. In: Cell Culture in the Neurosciences (Bottenstein JE, Sato G, eds). New York:Plenum Press, 223–243.

Honegger P, Matthieu JM. 1985. Aggregating brain cell cultures: a model to study meylination and demeylination. In: Cellular and Molecular Biology of Meylination (Jeserich G, Althaus HH, Waehneldt TV, eds). Berlin:Springer Verlag, 155–170.

Honegger P, Monnet-Tschudi F. 2001. Aggregating neural cell cultures. In: Protocols for Neural Cell Culture. (Fedoroff S, Richardson A, eds). 3rd ed. Ottawa:Humana Press, 199–218.

Honegger P, Richelson E. 1979. Neurotransmitter synthesis, storage and release by aggregating cell cultures of rat brain. Brain Res 162:89–101.

Hong MS, Hong SJ, Barhoumi R, Burghardt RC, Donnelly KC, Wild JR, et al. 2003. Neurotoxicity induced in differentiated SK-N-SH-SY5Y human neuroblastoma cells by organophosphorus compounds. Toxicol Appl Pharmacol 186:110–118.

Howard AS, Bucelli R, Jett DA, Bruun D, Yang D, Lein PJ. 2005. Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. Toxicol Appl Pharmacol 207:112–124.

Hutson LD, Chien CB. 2002. Wiring the zebrafish: axon guidance and synaptogenesis. Curr Opin Neurobiol 12:87–92.

Imitola J, Raddassi K, Park KL, Mueller FJ, Nieto M, Teng YD, et al. 2004. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci USA 101:18117–18122.

International Programme on Chemical Safety. 1996. OECD Screening Information DataSet (SIDS) High Production Volume Chemicals (Processed by UNEP Chemicals). Available: http://www.inchem.org/pages/sids.html [accessed 5 May 2006].

Ishikawa Y, Kage T, Yamamoto N, Yoshimoto M, Yasuda T, Matsumoto A, et al. 2004. Axonogenesis in the medaka embryonic brain. J Comp Neurol 476:240–253.

Jeram S, Riego Sintes JM, Halder M, Baraibar Fentanes J, Sokull-Klüttgen B, Hutchinson TH. 2005. A strategy to reduce the use of fish in acute ecotoxicity testing of new chemical substances notified in the European Union. Regul Toxicol Pharmacol 42:218–224.

Jin Y. 2002. Synaptogenesis: insights from worm and fly. Curr Opin Neurobiol 12:71–79.

Jones C, Reifegerste R, Moses K. 2006. Characterization of *Drosophila* mini-me, a gene required for cell proliferation and survival. Genetics 173:793–808.

Kinsner A, Pilotto V, Deininger S, Brown GC, Coecke S, Hartung T, et al. 2005. Inflammatory neurodegeneration induced by lipoteichoic acid from *Staphylococcus aureus* is mediated by glia activation, nitrosative and oxidative stress, and caspase activation. J Neurochem 95:1132–1143.

Komuniecki RW, Hobson RJ, Rex EB, Hapiak VM, Komuniecki. 2004. PRBiogenic amine receptors in parasitic nematodes: what can be learned from *Caenorhabditis elegans*? Mol Biochem Parasitol 137:1–11.

Krause G, Lehmann S, Lehmann M, Freund I, Schreiber E, Baumann W. 2006. Measurement of electrical activity of long-term mammalian neuronal networks on semiconductor neurosensor chips and comparison with conventional microelectrode arrays. Biosens Bioelectron 21:1272–1282.

Lambert DG, Nahorski SR. 1990. Second-messenger responses associated with stimulation of neuronal muscarinic receptors expressed by human neuroblastoma SH-SY5Y. Prog Brain Res 84:31–42.

Lein P, Johnson M, Guo X, Rueger D, Higgins D. 1995. Osteogenic protein-1 induces dendritic growth in rat sympathetic neurons. Neuron 15:597–605.

Leitner Y, Goez H, Gull I, Mesterman R, Weiner E, Jaffa A, et al. 2004. Antenatal diagnosis of central nervous system anomalies: can we predict prognosis? J Child Neurol 19:435–438.

Lettre G, Hengartner MO. 2006. Developmental apoptosis in *C. elegans*: a complex CEDnario. Nat Rev Mol Cell Biol 7:97–108.

Levitt P, Moore RY, Garber B. 1976. Selective cell association of catecholamine neurons in brain aggregates *in vitro*. Brain Res 111: 311–320.

Li J, Spletter ML, Johnson DA, Wright LS, Svendsen CN, Johnson JA. 2005. Rotenone-induced caspase 9/3-independent and -dependent cell death in undifferentiated and differentiated human neural stem cells. J Neurochem 92:462–476.

Liu MY, Hsieh WC, Yang BC. 2000. *In vitro* aberrant gene expression as the indicator of lead-induced neurotoxicity in U-373MG cells. Toxicology 147:59–64.

Ma W, Fitzgerald W, Liu QY, O'Shaughnessy TJ, Maric D, Lin HJ, et al. 2004. CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. Exp Neurol 190:276–288.

Margioris AN, Venihaki M, Stournaras C, Gravanis A. 1995. PC12 cells as a model to study the effects of opiods on normal and tumoral adrenal chromaffin cells. Ann NY Acad Sci 771:166–172.

Martinez-Contreras A, Huerta M, Lopez-Perez S, Garcia-Estrada J, Luquin S, Beas Zarate C. 2002. Astrocytic and microglia cells reactivity induced by neonatal administration of glutamate in cerebral cortex of the adult rats. J Neurosci Res 67:200–210.

Milosevic J, Schwarz SC, Krohn K, Poppe M, Storch A, Schwarz J. 2005. Low atmospheric oxygen avoids maturation, senescence and cell death of murine mesencephalic neural precursors. J Neurochem 92:718–729.

Monnet-Tschudi F. 1998. Induction of apoptosis by mercury compounds depends on maturation and is not associated with microglial activation. J Neurosc Res 53:361–367.

Monnet-Tschudi F, Zurich MG, Honegger P. 1997. Aggregate cell cultures for neurotoxicity testing: the importance of cell-cell interactions. Dev Animal Vet Sciences 27:641–649.

Monnet-Tschudi F, Zurich MG, Pithon E, Van Melle G, Honegger P. 1995a. Microglial responsiveness as a sensitive marker for trimethyltin (TMT) neurotoxicity. Brain Res 690:8–14.

Monnet-Tschudi F, Zurich MG, Riederer BM, Honegger P. 1995b. Effects of trimethyltin (TMT) on glial and neuronal cells in aggregate cultures: dependence on the developmental stage. Neurotoxicology 16:97–104.

Monnet-Tschudi F, Zurich MG, Schilter B, Costa LG, Honegger P. 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and gial markers in aggregating brain cell cultures. Toxicol Appl Pharmacol 165:175–183.

Neumann H, Boucraut J, Hahnel C, Misgeld T, Wekerle H. 1996. Neuronal control of MHC class II inducibility in rat astrocytes and microglia. Eur J Neurosci 8:2582–2590.

OECD (Organisation for Economic Co-operation and Development). 2006. OECD Guideline for the Testing of Chemicals. Draft Proposal for a New Guideline 426. Developmental Neurotoxicity Study. Available: http://www.oecd.org/dataoecd/20/52/37622194.pdf [accessed 1 October 2006].

Olney JW. 2002. New insights and new issues in developmental neurotoxicology. Neurotoxicology 23(6):659–668.

Orger MB, Gahtan E, Muto A, Page-McCaw P, Smear MC, Baier H. 2004. Behavioral screening assays in zebrafish. Methods Cell Biol 77:53–68.

Overstreet LS, Pasternak JF, Colley PA, Slater NT, Trommer BL. 1997. Metabotropic glutamate receptor mediated long-term depression in developing hippocampus. Neuropharmicology 36:831–844.

Pahlman S, Mamaeva S, Meyerson G, Mattsson ME, Bjelfman C, Hammerling U. 1990. Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. Acta Physiol Scan Suppl 592:25–37.

Park HC, Kim CH, Bae YK, Yeo SY, Kim SH, Hong SK. 2000. Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. Dev Biol 227: 279–293.

Parmar D, Yadav S, Dayal M, Johri A, Dhawan A, Seth PK. 2003. Effect of lindane on hepatic and brain cytochrome P450s and influence of P450 modulation in lindane induced neurotoxicity. Food Chem Toxicol 41:1077–1087.

Parran DK, Mundy WR, Barone S Jr. 2001. Effects of methylmercury and mercuric chloride on differentiation and cell viability in PC12 cells. Toxicol Sci 59:278–290.

Peden KW, Rutkowski JL, Gilbert M, Tennekoon GI. 1990. Production of Schwann cell lines using a reulated oncogene. Ann NY Acad Sci 605:286–293.

Peterson RT, Link BA, Dowling JE, Schreiber SL. 2000. Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc Natl Acad Sci USA 97:12965–12969.

Pinzon-Duarte G, Arango-Gonzalez B, Guenther E, Kohler K. 2004. Effects of brain-derived neurotrophic factor on cell survival, differentiation and patterning of neuronal connections and Muller glia cells in the developing retina. Eur J Neurosci 19:1475–1484.

Pittman RN, Wang S, DiBenedetto AJ, Mills JC. 1993. A system for chracterizing cellular and molecular events in programmed neuronal cell death. J Neurosci 13:3669–3680.

Prieto P, Blaauboer B, de Boer AG, Boveri M, Cecchelli R, Price A, et al. 2004. Blood–brain barrier *in vitro* models and their application in toxicology. The report and recommendations of ECVAM workshop 49. ATLA 32:37–50.

Ray DE. 1999. Toxic cell damage. In: Neurotoxicology *In Vitro* (Pentreath VD, ed). Philadelphia:Taylor & Francis, 77–104.

Reichel A, Begley DJ, Abbott NJ. 2003. An overview of *in vitro* techniques for blood-brain barrier studies. Methods Mol Med 89:307–324.

Rodier PM. 1995. Developing brain as a target for neurotoxicity. Environ Health Perspect 103:73–76.

Sachana M, Flaskos J, Alexaki E, Hargreaves AJ. 2001. Inhibition of neurite outgrowth in N2a cells by leptophos and carbaryl: effects on neurofilament heavy chain, GAP-43 and HSP-70. Toxicol *In Vitro* 15:115–120.

Sah DW, Matsumoto SG. 1987. Evidence for serotonin synthesis, uptake, and release in dissociated rat sympathetic neurons in culture. J Neurosci 7(2):391–399.

Sales KM, Kingston ST, Doyle KM, Purcell WM. 2004. Preliminary chracterization of an *in vitro* paradigm for the study of the delayed effects of organophosphorous compounds: hen embryo brain spheroids. Toxicology 195:187–202.

Sass JB, Haselow DT, Silbergeld EK. 2001. Methylmercury-induced decrement in neuronal migration may involve cytokine-dependent mechanisms: a novel method to assess neuronal movement *in vitro*. Toxicol Sci 63(1):74–81.

Seeds NW, Vater AE. 1971. Synaptogenesis in reaggregating brain cell culture. Proc Natl Acad Sci USA 68:3219–3222.

Seidman KJ, Teng AL, Rosenkopf R, Spilotro P, Weyhenmeyer JA. 1997. Isolation, cloning and characterization of a putative type-1 astrocyte cell line. Brain Res 753(1):18–26.

Shastry P, Basu A, Rajadhyaksha MS. 2001. Neuroblastoma cell lines—a versatile *in vitro* model in neurobiology. Int J Neurosci 108(1–2):109–126.

Stokes EA, Lonergan W, Weber LP, Janz DM, Poznanski AA, Balch GC, et al. 2004. Decreased apoptosis in the forebrain of adult male medaka (*Oryzias latipes*) after aqueous exposure to ethinylestradiol. Comp Biochem Physiol C Toxicol Pharmacol 138(2):163–167.

Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37(2):173–182.

Strasser U, Fischer G. 1995. Quantitative measurement of neuronal degeneration in organotypic hippocampal cultures after combined oxygen/glucose deprivation. J Neurosci Methods 57(2):177–186.

Takahashi J, Palmer TD, Gage FH. 1999. Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J Neurobiol 38(1):65–81.

Tiffany-Castiglioni E. 2004. *In vitro* neurotoxicology: introduction to concepts. In: *In Vitro* Neurotoxicology: Principles and Challenges (Methods in Pharmacology and Toxicology) (Tiffany-Castiglioni E, Hollinger, Mannfred A, eds). Totowa, NJ:Humana Press, 1–29.

Toda H, Takahashi J, Mizoguchi A, Koyano K, Hashimoto N. 2000. Neurons generated from adult rat hippocampal stem cells form functional glutamatergic and GABAergic synapses *in vitro*. Exp Neurol 165(1):66–76.

Turka LA, Goodman RE, Rutkowski JL, Sima AA, Merry A, Mitra RS. 1995. Interleukin 12: a potential link between nerve cells and the immune response in inflammatory disorders. Mol Med 1(6):690–699.

U.S. EPA (Environmental Protection Agency). 1998. High Production Volume Challenge Program (HPV). Available: http://www.epa.gov/hpv/ [accessed 7 May 2007].

van den Heuvel S. 2005. The *C. elegans* cell cycle: overview of molecules and mechanisms. Methods Mol Biol 296:51–67.

van Pelt J, Vajda I, Wolters PS, Corner MA, Ramakers GJ. 2005. Dynamics and plasticity in developing neuronal networks *in vitro*. Prog Brain Res 147:173–188.

Veronesi B. 1992. *In vitro* screening batteries for neurotoxicants. Neurotoxicology 13(1):185–195.

Volovitch M, le Roux I, Joliot AH, Bloch-Gallego E, Prochiantz A. 1993. Control of neuronal morphogenesis by homeoproteins: consequences for the making of neuronal networks. Perspect Dev Neurobiol 1(3):133–138.

Whiting PJ, Schoepfer R, Swanson LW, Simmons DM, Lindstrom JM. 1987. Functional acetylcholine receptor in PC12 cells reacts with a monoclonal antibody to brain nicotinic receptors. Nature 327:515–518.

Wiggin GR, Fawcett JP, Pawson T. 2005. Polarity proteins in axon specification and synaptogenesis. Dev Cell 8(6):803–816.

Windrem MS, Nunes MC, Rashbaum WK, Schwartz TH, Goodman RA, McKhann G II, et al. 2004. Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. Nat Med 10:93–97

Wittbrodt J, Shima A, Schartl M. 2002. Medaka: a model organism from the Far East. Nat Rev Genet 3(1):53–64.

Wullimann MF, Knipp S. 2000. Proliferation pattern changes in the zebrafish brain from embryonic through early postembryonic stages. Anat Embryol 202(5):385–400.

Yamamoto M, Urakubo T, Tominaga-Yoshino K, Ogura A. 2005. Long-lasting synapse formation in cultured rat hippocampal neurons after repeated PKA activation. Brain Res 1042(1):6–16.

Yoon MS, Yon C, Park SY, Oh DY, Han AH, Kim DS, et al. 2005. Role of phospholipase D1 in neurite outgrowth of neural stem cells. Biochem Biophys Res Commun 329(3):804–811.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. 2001. *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19:1129–1133.

Zimmer J, Kristensen BW, Jakobsen B, Noraberg. 2000. Excitatory amino acid neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures. Amino Acids 19(1):7–21.

Zurich MG, Eskes C, Honegger P, Berode M, Monnet-Tschudi F. 2002. Maturation-dependent neurotoxicity of lead acetate *in vitro*: implication of glial reactions. J Neurosci Res 70:108–116.

Zurich MG, Honegger P, Schilter B, Costa LG, Monnet-Tschudi F. 2004. Involvement of glial cells in the neurotoxicity of parathion and chlorpyrifos. Toxicol Appl Pharmacol 201:97–100.