

Compound selection for *in vitro* modeling of developmental neurotoxicity

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1. ABSTRACT

Development of *in vitro* systems, such as those based on embryonic stem cell differentiation, depends on the selection of adequate test and training compounds. We recommend the use of two classes of positive controls, the “gold standard compounds” for which developmental neurotoxicity (DNT) has been proven in man, and the “pathway compounds” that are known to disrupt signaling pathways and key processes relevant for neuronal

differentiation. We introduce the concept of toxicity endophenotypes (TEP) as changes in neuronal connectivity resulting from exposure to developmental toxicants. Thus, TEPs provide the scientific rationale for modeling DNT with simple *in vitro* models of key neurodevelopmental events. In this context, we discuss scientific and technical aspects of the test compound selection process. We suggest to include compounds with unspecific toxicity, besides negative control compounds, and we recommend tandem approaches to determine relative toxicities instead of

absolute measures. Finally, we discuss how to avoid pitfalls by distinguishing between unspecific forms of cytotoxicity and specific developmental neurotoxicity. A compilation of compound lists corresponding to the above-discussed principles supplement this review.

2. INTRODUCTION

Only few chemical substances in our environment and in consumer products are fully characterized for their toxicity. Developmental neurotoxicity (DNT) is currently the least examined form of toxicity, and is tested with OECD TG 426 (1). A recently published survey reports testing of about 100 substances, mainly pesticides. Another study reported neurobehavioral risk assessment for 174 compounds (1, 2). Apart from this small group, the majority of chemicals in our environment has not been tested for DNT (3).

DNT is also one of the most difficult forms of toxicity to pinpoint, as it is not necessarily related to cell loss. A change in the overall proportions of neural cells in the nervous system suffices to alter its function significantly. Similarly, changes in positioning, organization and connectivity of any given number of neurons affect their network function. The resultant forms of DNT, such as behavioral problems and speech impairments are difficult to model in animals and *in vitro* systems (4).

Only for a handful of chemicals such as some heavy metals (arsenic, lead, mercury), polychlorinated biphenyls (PCBs), solvents (alcohol, toluene), and a few pesticides, a clear association with human DNT has been shown in epidemiological studies (3, 5). For about 100 additional chemicals, developmental toxicity may be inferred from animal studies (6).

However, regularly drugs emerge that have passed extensive animal testing (including primate testing) and fail in clinical trials due to unexpected adverse effects. There is no reason to believe that for DNT these interspecies differences would be irrelevant. Moreover, the human nervous system is larger in size and complexity (e.g. pre-frontal cortex) than that of any other species and requires years to fully mature. It is therefore likely more susceptible to perturbation. Human cell-based DNT test systems avoid the need for species extrapolation. First attempts have been undertaken to model the neurobehavioral changes induced by DNT compounds by using *in vitro* tests (7). Following the National Research Council (NRC) initiative on toxicology for the 21st century (21c-Tox) new *in vitro* methods have been developed and may be assembled to a tiered battery of tests capable of improving the predictive value of toxicity testing with respect to human health (8).

The advent of embryonic stem cells (ESCs) allows for the first time to develop novel test systems modeling early human neural development *in vitro*, thus enabling this aspect of DNT testing (9, 10). It has been shown for murine embryonic stem cells (mESCs) that the

differentiating cells followed the main stages and transitions of *in vivo* embryonic development (11). When analyzing gene expression patterns underlying these events, it was shown that the differentiating mESC transit through a stage of neuroepithelial progenitors displaying characteristics of their embryonic counterparts, including polarity, Notch signaling, and appropriate timing of arising of neuronal and glia cells. By using embryo-oriented criteria during data analysis it emerged that differentially expressed genes included genes for primitive ectoderm, followed by gene signatures for neural progenitor development, neural induction and final terminal differentiation (12, 13). While similar formal proof is lacking for human embryonic stem cells (hESCs), it is assumed that similar to mESC, hESC recapitulate human embryonic/fetal neural development. Accordingly, hESC are increasingly used to investigate human early embryonic development (14, 15). For instance, disturbance of neural differentiation of hESC by retinoic acid (RA) resulted in similar morphological and transcriptional changes as observed during fetal development *in vivo* (16).

To successfully use hESC-derived cells for *in vitro* assays for DNT, careful assessment of the test systems is crucial. This includes foremost a selection of endpoint-selective and training compounds, to test whether the system performs as expected, and to determine appropriate end-points, read-outs, and the dynamic range of responses of the system (6, 17). The specificity, selectivity and the predictive value of a test system can only be determined after a set of compounds has been tested. We here summarize several considerations that may inform this non-trivial task.

3. NEURAL DEVELOPMENT AND EMBRYONIC STEM CELLS

The development of a complex vertebrate organism requires several tightly regulated and exactly timed processes. Particularly, the development of the sophisticated mammalian nervous system requires a precisely orchestrated sequence of key developmental events including proliferation, cell migration, patterning, apoptosis, cell differentiation, neurite outgrowth, synaptogenesis and pruning, myelination and neurotransmitter turnover (Figure 1).

The nervous system is composed of different cell types such as glial cells (e.g. astrocytes, Schwann cells) and different subtypes of neurons that are generated via differentiation of multipotent stem cells during development (18). During the development of the nervous system, these processes are exquisitely time and region-specific, and as neural tube closure proceeds both towards the anterior and posterior end of the embryo, similar region-specific processes occur in parallel at different areas of the embryo (19). Thus, neural development is a tightly orchestrated continuum in which the same, as well as different, processes occur at different locations at different times or simultaneously, from the anterior (rostral) tip of the embryo to the tail (caudal) end.

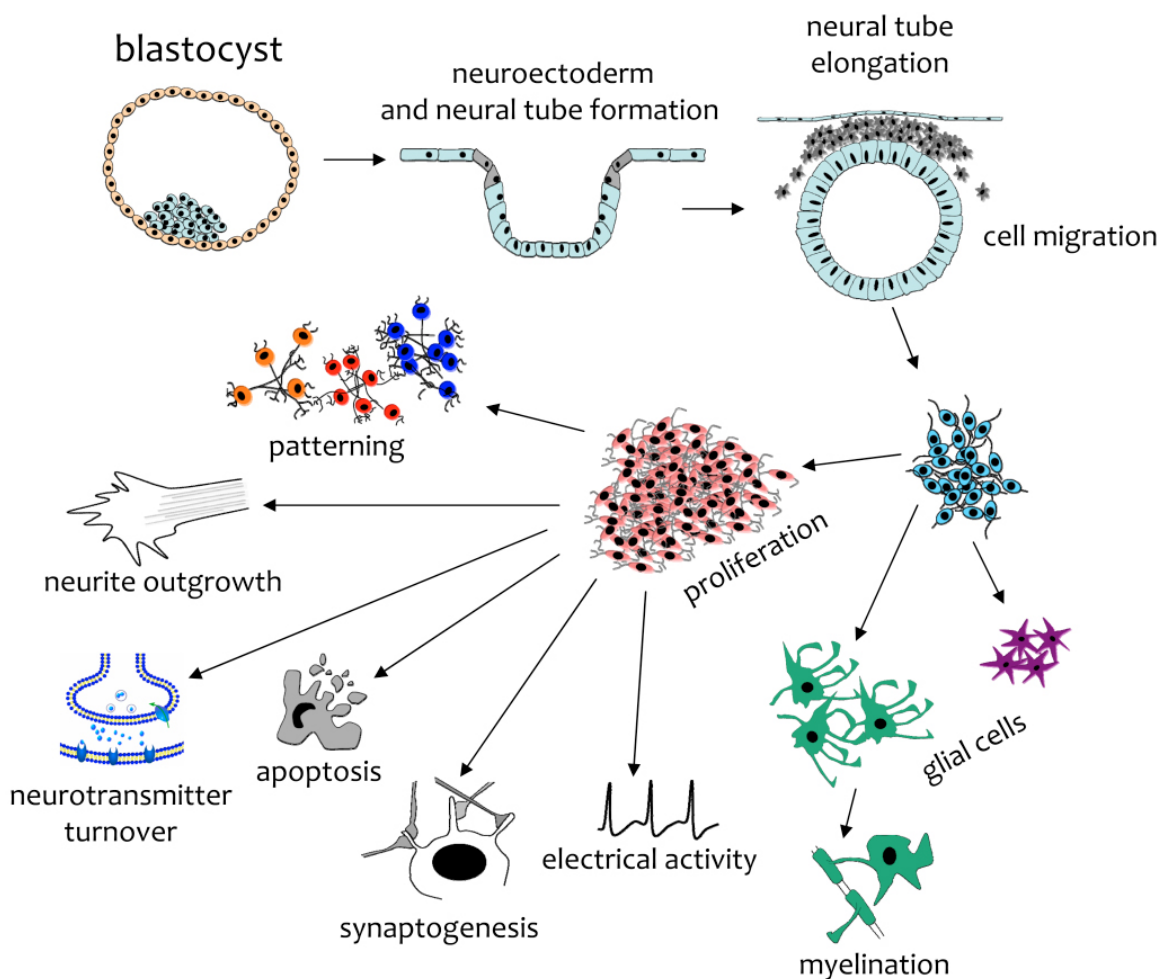


Figure 1. Schematic representation of processes involved in neural differentiation. During gastrulation, neurulation starts with the formation of the neuroectoderm, neural plate and subsequent formation of the neural tube. During these events, migration of NPCs (CNS) and neural crest cells (PNS) takes place as cells migrate to their destination. Cells undergo differentiation, apoptosis and patterning. Once at their destination, newly born neurons extend neurites and form synapses.

Since the establishment of the first murine (mESC) and human embryonic stem cell (hESC) lines, much effort has focused on establishing and optimizing neural differentiation protocols to generate different types of neural cells, for regenerative medicine and more recently, also for disease modeling (11, 20-24). Differentiating ESCs recapitulated first neurulation, then anterior/posterior and dorso/ventral patterning, followed by neurogenesis and last gliogenesis, in a time line close to *in vivo* embryonic development, with similar wave-like gene expression patterns (11, 25). For this reason, more and more stem cell-based differentiation systems are used to establish screening assays for drug discovery and toxicological studies (26-29). These systems model several different aspects and stages of neurodevelopmental processes. One of the significant advantages of ESC-derived models is also the capability to model early neural development, i.e. the generation of very early neural precursor cells (NPCs) from ESCs. This allows studying toxicity mechanisms during the generation of these crucial cells, with almost pure

populations of NPCs. *In vivo*, such detailed studies on generation of NPCs are complicated by the difficulty of identifying effects on very few cells within a quickly changing and developing early embryo. Mechanisms regulating the birth of early NPCs are therefore difficult to investigate *in vivo*.

4. THE ROLE OF „TOXICITY ENDOPHENOTYPES (TEP) AND KEY NEURO-DEVELOPMENTAL PROCESSES FOR THE CLASSIFICATION OF TOOL COMPOUNDS

4.1. The sequence of events connecting initial chemical insult with later behavioral phenotype

Chemical exposure during development is assumed to affect cellular and molecular structures. This would trigger biological processes leading to the impairment of one or several key neurodevelopmental processes (e.g. impaired migration or myelination) (Fig.1). At later time points, when such processes themselves

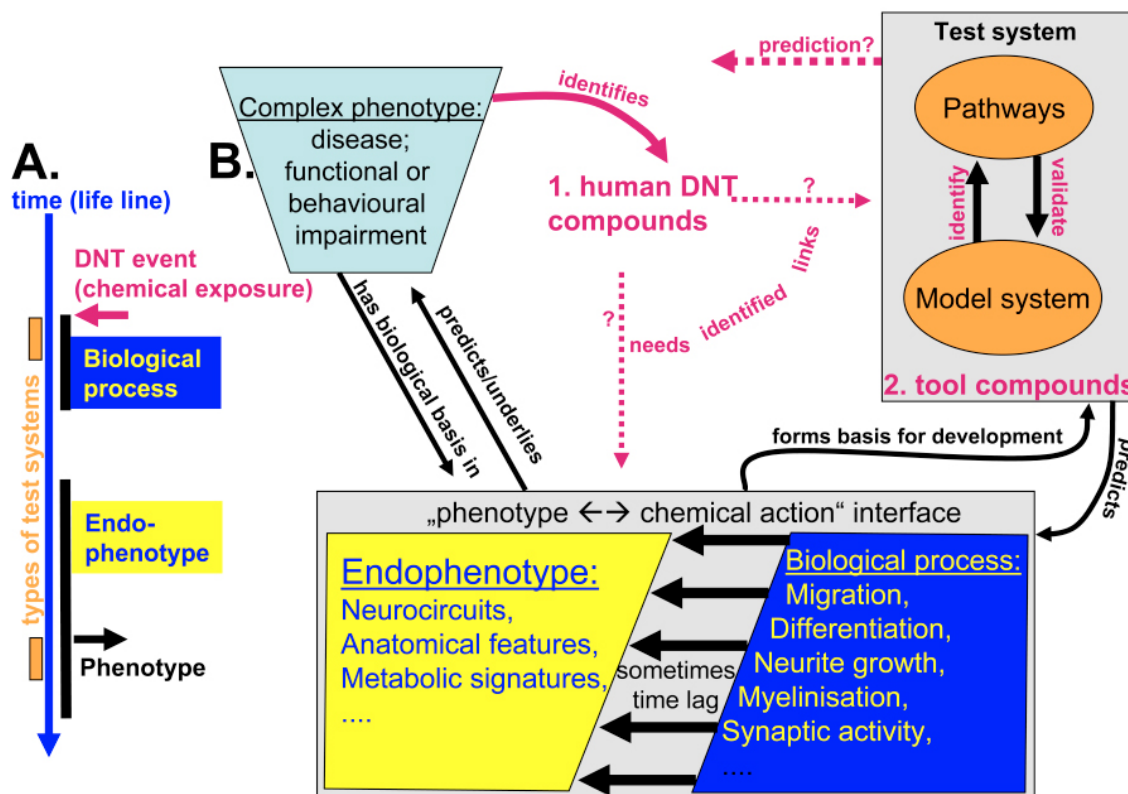


Figure 2. Endophenotypes as links between model systems and human DNT define two classes of test chemicals for model set-up. A. Illustration of the time line of DNT-relevant events. Note that the chemical trigger often precedes the observed clinical phenotype by long time spans (up to decades). B. Illustration of endophenotypes and underlying biological processes as interface between chemical action and the resultant clinical phenotype. The implications for different types of test compounds (1. human DNT compounds; 2. tool compounds) are indicated (purple colours).

possibly play no role anymore in the brain, this may result in altered neuronal connectivity. Models for such changes in connectivity have been suggested in other clinical areas, and they may range from altered synaptic density to a disorganized brain structure (30). Such alterations would affect neuronal circuits, and thereby result in a clinically measurable phenotype (e.g. impaired cognitive ability), also termed “exophenotype” or “apical endpoint of toxicity” (Fig.2).

4.2. Design of test systems, and their biological justification

The biological rationale for the use of *in vitro* test systems is that they model part of the sequence described above. A major challenge lies in linking the initial parts of this chain of events to the later ones. One potential approach is the use of defined molecular alterations, such as NMDA receptor function that may be traced through different levels of complexity (7). Most frequently, test systems model the initial events. In these cases, the test system endpoints cannot and do not address the clinical phenotype. Instead, they use molecular and cellular endpoints that are thought to be important for the establishment of neuronal network function. It is important to distinguish this situation from the one in which the test system directly models a later event (e.g. disturbed

neuronal connectivity). Such latter test systems do not model the originally disturbed biological processes, but their result in the developed brain (Figure 2).

Another approach to link *in vitro* test systems to the final outcome of DNT may be based on the use of pathway-specific tool compounds or chemicals with well-characterized modes of action. Examples of such compounds are certain organophosphate compounds or PCBs (7). Their actions may be followed in models of increasing complexity ranging from primary neuronal cultures to experimental animals. The effects in *in vitro* systems may be linked to alterations in connectivity, and these may be eventually linked to behavioral endpoints also relevant for man.

4.3. Description of changes in neuronal connectivity as toxicity endophenotypes (TEP)

Endophenotypes have been originally defined in psychiatry. We suggest here “toxicity endophenotypes (TEP)” (see Figure 2) as a similar, yet not identical, concept for DNT. In psychiatry, endophenotypes are considered as “measurable components along the pathway between distal genotype and disease”. The definition excludes gross histopathological changes, but refers to changes in microanatomy, neuronal connectivity and

neuronal physiology leading to altered neuronal network functions. An endophenotype may thus be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, or neuropsychological (including configured self-reported data) in nature. For practical reasons, they are linked to the methodology used for their measurement, and they have emerged as an important concept in the study of complex neuropsychiatric diseases (e.g. schizophrenia or autism) (31-34).

What can be gained by using the concept of TEP? Developmental neurotoxicity (DNT) usually results in a complex phenotype, comparable to complex phenotypes in psychiatric disease. In both cases, these complex phenotypes are difficult to link to the initial causes. The reason may for instance be that different initial triggers may lead to similar external symptoms. Moreover, the external phenotype is a combination of different symptoms that may have different underlying biological causes. The concept of endophenotypes has been used in the field of psychiatric disorders to avoid the problem of linking the complex "external" phenotype to underlying genetic changes. Instead of the external phenotype, more sharply defined endophenotypes have been used successfully to identify genetic associations. Prepulse inhibition (PPI) for instance is regarded as an endophenotype for schizophrenia (35). With the help of the concept of endophenotypes, initial genetic risk factors such as neuregulin-1 polymorphisms have been related to this neuropsychiatric disease (36). Similarly, specific language abnormalities are an endophenotype of autism, and they have been linked to the CNTNAP2 gene (37). We propose to extend the concept of endophenotypes to the field of DNT, however with a slightly altered definition. It is suggested to define toxicity endophenotype (TEP) in DNT as "endogenous biological (anatomic, genetic, functional, neurochemical) changes resulting from the initial chemical adverse effect, and thus representing a link between chemical insult and complex phenotype".

4.4. TEP as link between behavioral endpoints and initial molecular events triggered by chemicals

The complex phenotype of DNT is a result of initial molecular and cellular biological processes triggered by chemicals which then lead to delayed and possibly stable alterations in brain structure or function, described by the TEP. TEP are conceptually similar to "changed neuronal connectivity", but they are defined more operationally by the method to identify them (e.g. MRI scanning, electroencephalography, histology). An example for a TEP may be disorganization of cortical layer IV. The biological basis for this may be disturbed migration at an earlier time point of development (e.g. by chemically disturbed integrin signaling), or an imbalance of cell death and proliferation (e.g. by anti-apoptotic activity of a chemical specifically pronounced in a particular cell type). Such processes are non-exclusive, and may be defined at multiple levels of biological complexity. The molecular and cellular changes underlying the biological processes, but not the complex phenotype itself, can be modeled in cellular test systems. Thus, the TEP and their underlying key neurodevelopmental biological processes represent the

interface between test systems and expected human phenotypes.

Human DNT compounds identified by epidemiological methods need to be linked to TEP/biological processes and their underlying molecular and cellular changes in order to inform on the performance of a test system. Endpoint-specific tool compounds with known modes of action on defined biological pathways can be used to translate knowledge on TEP into useful test systems. They may also be used to identify toxicity pathways relevant for such models or to validate the reflection of such pathways by the models. The ultimate challenge lies in linking predictions from the test systems on unknown test compounds to their significance for complex DNT phenotypes. Potential paths towards this goal comprise large scale correlative efforts and the closing of the knowledge gap on the relation of model systems to relevant TEP and biological processes. In this context, it is important to note that there is a time offset of cause and effect in DNT.

4.5. Concepts related to TEP

Similar to TEP, other descriptions of intermediary effects are under discussion, and represent a dynamic area of current concept development in toxicology. The concept of pathways of toxicity (PoT) has its basis in the National Academy of Sciences report on a new vision for toxicology of the 21st century (38-40). The concept of PoT focuses strongly on cellular and molecular changes resulting from chemical injury. The extrapolation to complex neurobehavioral readouts is not yet clear, but the concept promises a very comprehensive coverage of many different toxicity domains. The OECD operates with the concept of adverse outcome pathways (AOPs), which has been elaborated in great detail especially in the area of ecotoxicology (41), but also for neurotoxicology (42). The concept is quantitatively more elaborate than the simple construct of TEP, and it has already been used to link chemical target effects via intermediate actions to disturbances of whole ecosystems. PoT and AOP concepts have been designed to model the unidirectional linkage from chemical molecular changes to more complex cellular or organismal disturbances. Endophenotypes have been historically used to work backwards (in the opposite direction), to identify molecular causes for complex pathologies. In the context of test system definition and tool compound selection all approaches have their merits. The concept of TEP is the simplest approach, and in contrast to AOPs and PoTs, it will not be useful for quantitative predictions. However, it provides a particularly strong focus on the issue of intermediate system changes between molecular events and apical toxicity endpoints, and it directs the attention particularly to the importance of the time offset component in such changes.

5. PROMISING ENDPOINTS FOR *IN VITRO* DNT TESTING

Gene expression is one of the most commonly used endpoints to detect effects of compounds on stem cells differentiating into neural cells (16, 26-28, 43). Gene

expression after exposure to methylmercury was also used to investigate the later stages of neuronal maturation that include establishing the appropriate neurotransmitter balance and development of the dopaminergic system. As reported previously (7, 44), gene expression endpoints and functional readouts were correlated to molecular changes and neurobehavioral effects known from *in vivo* models (45-47). Other important targets of neurodevelopmental toxicity, such as differentiation of cells, neurite outgrowth or migration of neural progenitor cells have also been successfully modeled *in vitro* (48-53). Such functional endpoints could be used as the endpoints of choice when establishing new *in vitro* toxicological screening assays.

To be able to implement these important mechanistic endpoints in the test systems under development, it is very important to know detailed characteristics of the system such as gene expression profiles, which biological process one should/can model, and which biological and molecular pathways are present and functional in the *in vitro* system chosen.

6. GENERAL PRINCIPLES FOR DEVELOPING AN *IN VITRO* MODEL FOR DNT

6.1. Biological characterization of the system prior to training compound choice

One central characterization approach involves a careful characterization of gene expression profiles in the cell culture system over time. Data on gene expression profiles during the differentiation process will not only verify that the differentiation process proceeds according to expectations, but will also set the stage for identification of useful markers to analyze during training compound testing. One may attempt to define the time point at which the culture transits through a stage of highly enriched neural precursors, when the culture is in the process of developing neuronal/glial precursor cells or when the culture is beyond the branching point between neuronal and glial development. Gene expression profiles may be particularly valuable if they are correlated to defined biological processes (25).

6.2. Key neurodevelopmental processes that can be modeled *in vitro*

6.2.1. Cell division, cell death and proliferation

Stem cell cultures differentiating to more mature/specialized cell types will progress in a dynamic manner towards states of advanced and/or terminal differentiation. During this process, the cells will change their chromatin to enable transcriptional access to genes specific for the stages they are transiting through, and they will also divide several times. Later on, depending on their stage of differentiation, different processes like e.g. cell death may take place, cell division will slow down and cellular events resulting in expression of lineage-specific genes determining the cell type will take over. During corresponding *in vivo* processes, neuronal precursors migrate to their destination and acquire the phenotype of a particular neuronal lineage. At this stage, many of the arising neurons are subjected to massive cell death (54, 55). As cell death can also occur *in vitro* during the neurulation

stage, there can be strong cell division without any notable overall increase of cell numbers/mass (56).

It is therefore crucial to choose appropriate read-outs, as for example population measurements using resazurin reduction can only inform about total viable cell numbers, but not exact proportions of subpopulations. Effects of compounds resulting in the targeted death of certain subpopulations may not be appreciated in the global population with such classical toxicity read-outs due to compensatory growth/proliferation by other cells. For example when injected into 1 month old rats, methylazoxymethanol (MAM) killed only dividing cells in the brain but enhanced survival of progeny, resulting in generation of more neurons, through increased survival of new-born granule cells (57). A skewing towards one population or the other may be better detected by skewing of marker gene expression. Moreover, it needs to be noted that cell death mechanisms of developing neurons are different from those found in adult cells (58).

6.2.2. Migration

Cell migration is a crucial feature of neural development. CNS and PNS progenitor cells have to migrate along defined paths in a strictly timed manner to guarantee correct neural development (59, 60). This is accompanied by dynamic processes such as reorganization of the actin cytoskeleton and membrane compartments, rearrangement of the extracellular matrix (ECM) and cell junctions, and detachment/reattachment via adhesion molecules. Also, the cells have to be able to sense gradients of chemoattractants (e.g. CXCR4) or repellents (e.g. Sema3A). For this, the cells have to express the corresponding receptors at the appropriate time. Once the signal has been sensed, it has to be translated into a cellular response. In the case of migration, this is mainly done via members of the Rho family of small GTPases such as Rac, Rho or Cdc42, resulting in a reorganization of the actin cytoskeleton including actin polymerization, contraction via interaction with myosin and adhesion to the substrate, among others via integrins (61, 62). All these biological pathways can provide useful targets for tool compound selection when establishing an *in vitro* assay (for detailed reviews on cell migration during development see Kurosaka and Kashina, 2008 and Valiente and Marin, 2010 (59, 63).

6.2.3. Differentiation

The exact mechanisms involved in differentiation will be different from system to system, and even from cell type to cell type, but generally important signaling pathways have emerged. Some pathways that are important during early differentiation of ESCs to neural lineages include: BMP signaling repressing neural fate, Wnt signaling pathway affecting neural fate at most stages of neural development, Notch signaling, fibroblast growth factor-2 (FGF2) signaling to support neural stem cells formation/survival, and morphogens such as retinoic acid (RA), sonic hedgehog (SHH) and FGF8 to define anterior-posterior and dorso-ventral identity of neuronal subtypes (15, 64-69).

During the differentiation process, changes in mRNA expression go hand in hand with changes in the epigenetic profile, changes in micro RNA (miRNA) expression, and changes in metabolic profiles (70-72). Of particular interest are recent studies analyzing mRNA profiles during differentiation in the presence of toxicants (26, 28). Also of interest is a recent study identifying changes in metabolites during differentiation of ESCs in the presence of defined toxicants (73).

The populations generated in differentiating cultures may not at all stages display the same sensitivity to a chemical or signaling cue for lack of receptor expression, incomplete expression of signaling pathways, and/or incomplete expression of effector pathways. For instance, BMP2 triggers largely different responses at different phases of neuronal development (74). Also, differentiating cells may be more sensitive to certain compounds than their fully differentiated progeny. For example, methylmercury and lead trigger fetal neurotoxicity at concentrations not affecting the mother. Accordingly, when methylmercury *in vitro* toxicity was compared between developing neurons and fully differentiated neurons within the same ESC-derived system, EC50 concentrations of methylmercury cytotoxicity were 60 nM for developing neurons while for fully developed neurons the EC50 was as high as 9.5 μ M (44). Another difficulty of stage-specific toxicity is species extrapolation. For instance, postnatal days 1-10 in the rat correspond to the last trimester of human pregnancy (75). Similar data on *in vitro* systems are still sparse.

While several studies have addressed the inhibition of neuronal differentiation, less is known about chemical effects on gliogenesis. As the transcriptome of different types of glial cells is well characterized (76-78), and protocols for the generation of glia cells from ESC are emerging (79), more information on this process is expected in the future.

6.2.4. Neurite outgrowth

In the case of neuronal development, once progenitor cells have reached their target site they have to differentiate to fully mature neurons and generate the complex neurite network that is characteristic for the highly developed mammalian nervous system. Neurite outgrowth relies on intrinsic (e.g. expression of receptors) and extrinsic factors. An important extrinsic aspect is the interaction of the differentiating cells with components of the extracellular matrix (ECM) as well as with other cells such as glial cells via cell adhesion molecules (CAMs) and integrins (80-82). Similar to the processes already described for cell migration, gradients of attractants and repellents are sensed by the growth cone of growing neurites, leading to actin reorganization via GTPases and directed growth of the neurite. It has been proposed that the signals from the ECM or extracellular guidance cues resulting in neuron polarization converge at the level of GSK3 (83). Similar to cell migration, neurite outgrowth and polarization include multiple pathways that could be targeted by chemicals and tool compounds (47, 53).

6.2.5. Synaptogenesis

The mammalian brain comprises 10^{11} neurons, which are connected to each other by up to 10^{15} synapses (84). After the axonal growth cones have reached their targets, functional synapses have to be established. The process of synapse formation, called synaptogenesis, can be broken down into two major stages. First, initial contact is made between the two cells, and synapses are formed. Next, synaptic connectivity is fine-tuned by eliminating (pruning) as well as strengthening of synapses. For this, the synapse-forming and synapse-receiving cells exchange signals and initiate the second step of synaptogenesis. Dendrites as well as axon-specific protein complexes (active zone proteins, synaptic vesicle proteins) are recruited to the initial contact site and a functional synapse is formed (85, 86).

7. COMPOUND SELECTION STRATEGIES

7.1. Selection criteria

Different sets of tool compounds need to be selected depending on the aim of the assay one would like to develop. Thus, it is important to distinguish: (a) the initial setup of an assay, (b) the testing of assay function, (c) validation/blinded testing of the developed assay, (d) a biomarker identification study, or, (e) a validation study for the identified biomarkers. Compounds used for assay establishment have also been called “endpoint-specific controls” (6). Compounds, evaluating the performance of the assay after its establishment may accordingly be termed “training set”, and a “testing set” would be used to examine how far the assay response reflects the human or animal data. In most parts of this review, selection criteria for (a) and (b) are proposed:

- The list of reference compounds should cover a range of chemical classes. The type of classes depends on the assay in development e.g. a screening assay for pharmaceutical industry should include drugs as tool compounds. If an assay is developed to test environmental toxicants inclusion of metals and pesticides is advisable.
- Ideally, the information on the toxicity profiles of the chosen substances should be comprehensive, providing human and animal data, mechanistic information, and data from other test systems (e.g. derived from PubChem, ACTOR [<http://actor.epa.gov/actor>], or the OECD eChemPortal [<http://www.echemportal.org>]).
- Knowledge about the underlying toxic mechanisms should exist or hypotheses about the mode of action and the biological process affected should be deducible from scientific literature.
- This mechanistic knowledge should be, at least partly, derived from other *in vitro* tests using e.g. primary neurons, stem cells, neuronal cell lines (e. g. PC12, SH-SY5Y), or mechanistic test systems. If no *in vitro* information is available, potentially confounding factors (biokinetics, metabolism, indirect toxicity) need to be considered.
- During the first phase of assay development, compounds that need metabolic activation should be

excluded. Later in assay development such compounds and their pairing metabolite (see below) should be included to define the assay characteristics.

7.2. Tandem Approaches

In order to assist the evaluation of a new *in vitro* test system so called “tandem approaches” may be useful to test particular aspects of the developed assay. These approaches are based on relative comparison rather than on the use of absolute positive and negative controls.

7.2.1. Substance tandems

Toxicity is always a matter of concentration. At sufficiently high concentration even sugars become toxic. The substance tandem approach is based on pairing of a toxic compound with a non-toxic, or less toxic, chemical analogue to evaluate their relative differences in the test system. This then allows assessing the specificity of the test system, as it should detect the relative difference in toxicity. This approach also avoids the need to define absolute potencies and toxicity thresholds for ranking of compounds according to their toxicity. Instead, compounds are ranked relative to each other.

This concept has been successfully applied for valproic acid, a known developmental toxic compound, and different structural analogues (87). Similarly, this concept might be applied in metal toxicity. Mercury may be paired with other metals known to be less toxic to the developing nervous system, e.g. MgCl_2 .

For some pharmacologically active drugs, compound pairs are available. An example of such a pair is 2-Fluoro-N-[2-(2-methyl-1*H*-indol-3-yl)ethyl]-benzamide (CK-666), an inhibitor of the Arp2/3 complex (88) which plays a central role in nucleation of branched actin filament polymerization and the autophagic machinery (89). The corresponding inactive molecule is CK-689. Another pair of small molecule inhibitors is locostatin (UIC1005), an oxazolidinone derivative and inhibitor of Raf kinase inhibitor protein (RKIP) and its pair-compound UIC1017, a non-toxic molecule (90, 91).

Additionally, chemically related compound pairs such as n-hexane and 3-methylpentane, a chemically related isomer, that does not form the toxic metabolite 2,5-hexanedione, can be used. This tandem would require metabolic activity, which will be discussed later in detail.

7.2.2. Cell type tandems

Similar to the substance tandem concept, a sensitive cell type may be compared to a resistant cell type. If one cell type does not express certain receptors or pathways which are known to be triggered by a chosen substance, this cell will expectedly be resistant to the effects of the substance tested. Particular subtypes of neurons (e. g. dopaminergic neurons) might be more vulnerable to certain toxicants than others. For reference compounds such as manganese or 1-methyl-4-phenylpyridinium (MPP^+) two subtypes of neurons (e. g. sensitive dopaminergic vs. less sensitive GABAergic neurons) can be compared. A GABAergic neuron will not

die upon exposure to MPP^+ as it does not express the dopamine transporter required to internalize it, whereas a mature dopaminergic neuron should die, as it expresses the dopamine transporter. Such a set-up allows specifying the most appropriate read-outs to provide the most sensitive information about toxic effects. One way to conveniently generate such cell type tandems for mechanistic studies is the knock-down of presumed targets by small interfering RNAs.

7.2.3. Tandems of compounds in different oxidation or aggregation states

Especially for neurotoxic metals such as manganese, aluminum, or lead, the ionic state (valence state) of the neurotoxin might be relevant. Therefore, manganese(II)chloride (+2 valence state) might be paired with manganese(III)acetate or manganese(IV)phosphate. This pairing procedure might identify the cellular/molecular targets (e. g. transporter systems, ion channels, other membrane structures) underlying the toxic effects of the particular metal species. Important examples are also metal-based nanoparticles that may be compared to the same metal in soluble or large particulate form.

7.3. Limitations of *in vitro* tests due to metabolic effects, different defense mechanisms, and indirect effects

7.3.1. Metabolism

Metabolism of xenobiotics usually serves detoxification in the organism, but for some compounds metabolism generates neurotoxic metabolites. This process can take place either in non-neural tissue (e. g. the liver) but also in the brain. Thus, *in vitro* test systems lacking metabolizing activity can overestimate or underestimate the toxicity a compound would have *in vivo*. This is a known problem for test system design and has to be taken into account when choosing appropriate training compounds.

A standard compound used in the field of *in vivo* neurotoxicity, MPTP, exemplifies this. Upon injection into animals, MPTP crosses the blood brain barrier (BBB), and is metabolized within the brain by the astrocyte enzyme monoamine oxidase B (MAO-B) to generate the neurotoxic compound MPP^+ . MPP^+ can then be taken up into dopaminergic neurons via dopamine transporters. MPTP is not neurotoxic in systems that lack the above-mentioned metabolic capacity (92, 93).

On the other hand, there are also compounds which cause no neurotoxicity *in vivo*, but are neurotoxic *in vitro*. One such example is the metabolite of MPTP, MPP^+ . While MPTP easily crosses the BBB *in vivo*, MPP^+ cannot reach the neurons in the brain, as it does not cross the BBB. Injected into an animal, it would not be neurotoxic. However, in an *in vitro* system containing dopaminergic neurons expressing the dopamine transporter, it will be highly toxic.

Another example of a compound being toxified by metabolism (hepatic) is the neurotoxic metabolite of n-hexane, 2,5-hexanedione. Hexane itself has certain

anesthetic properties, but cannot be expected to show specific neurotoxicity in stem cell-based DNT test systems lacking the metabolizing capacity of liver cells. Other relevant examples of metabolized compounds include parathione (metabolized to paraoxone), statins in their lactone form (bioactive only as carboxylic acids), heroine (deacetylated to morphine), and retinol (active as retinoic acid).

7.3.2. Biological Barriers

There are several *in vivo* defense mechanisms such as the blood-brain barrier (BBB), the placental barrier (PB), the binding in blood (e. g. to erythrocytes as known for bromide, cyanide), or to other non-neural tissue (e. g. fat deposits), or the sequestration of compounds by metallothioneins (e. g. cadmium). On the other hand, there are also specific defense mechanisms in hESC, such as high expression of the multidrug transporter ABCG2 that protects the cells against xenobiotics (94). In both instances, the active site concentrations are hard to compare between *in vivo* and *in vitro* systems. In extreme cases, this might lead to situations as described above for metabolism. Loperamide, for instance, is an opioid receptor agonist widely used as antidiarrheal agent. However, its central neuronal effects are irrelevant *in vivo* because it does not cross the BBB (95). Thus, compounds of known action that are not neurotoxic or do not trigger DNT *in vivo* may still provide good positive controls for *in vitro* systems, where such biological limitations do not exist.

7.3.3. Indirect toxicity

When selecting compounds for an *in vitro* DNT assay, one has to distinguish direct from indirect toxic effects. *In vivo*, DNT may be triggered by many indirect effects. Maternal toxicity may affect the developing fetal brain. Toxicity to the thyroid, placenta or to functions important in feeding might adversely affect pre- and postnatal neural development. Indirect toxic effects may also be caused by interferences of chemicals with oxygen or glucose supply of the developing brain and the subsequent effects of these events on the developing neural tissue. Interferences with glucose circulation and the subsequent transport into the brain have been observed for neurotoxins like dichloroacetic acid and some compartments of the brain seem to be more vulnerable for this indirect mechanism (e. g. some thalamic nuclei) (96). Many astrocyte-specific toxicants affect energy metabolism (fluoroacetate, 6-aminonicotinamide, fluorocitrate). This can lead to secondary neurotoxicity. Other compounds affect oligodendrocytes or blood vessels, and trigger indirect neurotoxicity, that may be region-specific. A particularly interesting example is the insecticide fipronil which shows developmental neurotoxicity and notochord degeneration in zebrafish at high concentrations (0.7-1.1 μM). The hypothesis is that a block of glycine receptors leads to muscle cramps that are so strong that they damage the notochord. Thus, non-neural cells/organs are involved in the developmental neurotoxicity (97). Substances known to act predominantly via such indirect mechanisms are not suitable for the validation of any *in vitro* test systems.

8. TWO DIFFERENT CONCEPTS TO SELECT TOOL COMPOUNDS

8.1. “Gold standards” vs. “mechanistic tool compounds”

Work on the compilation of the reference compound list contained herein started with a bibliographic search for suspected developmental neurotoxins. Sources were: Grandjean and Landrigan, 2006, Spencer *et al.*, 2000, the HSDB[®] US National Library of Medicine (accessed at: <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>), and existing compound lists such as the 97 compounds used for ACuteTox, a compilation of suspected DNT compounds that were identified in animals and the 50 compounds (<http://www.cctoxconsulting.a.se/meic.htm>) used in the MEIC study (3, 6, 98-100).

While not much structured information is available on targeted compound selection for DNT, some literature is available on principles of compound selection itself. For instance, several EU-funded research consortia describe their procedure of compound selection in the area of carcinogenesis or reproductive toxicology (101, 102). These compilations are based mainly on information available from animal studies. A similar approach has been taken for the selection of DNT compounds and for selection of compounds in the ToxCast program (6, 103).

More recent studies focus on the identification of pathways of toxicity (PoT). The identification of pathways that are affected by specific toxicants is expected to enable the targeted identification of biomarkers (38). Accordingly, the use of compounds known to interfere with pathways expressed in the experimental system (endpoint-specific controls), has been recommended by the DNT2 and DNT3 conferences as initial step of system characterization (6, 104). The approach using specific pathway inhibitors should be complemented by the use of a set of unspecific toxicants to determine overall assay characteristics (17).

At present, two approaches for compound selection seem useful:

A. Use of compounds that are known to trigger DNT. These may be called “gold standard” compounds if the information is relevant to human, or if there is compelling evidence from several other DNT assays.

B. Use of compounds that are known to disrupt cellular processes that would be relevant for DNT, these could be called “mechanistic tool compounds”.

The advantage of using gold standard compounds is their toxicological relevance, as their role in human DNT has already been proven by epidemiological studies. Furthermore, the results obtained with the *in vitro* assay can be linked to existing knowledge in the human context. Such an approach has already been used successfully for the development of skin and ocular toxicity *in vitro* tests as well as in the field of reproductive toxicology.

The disadvantage of the gold standard compound approach is that in contrast to ocular and skin toxicity, only few DNT compounds are known. In ocular and skin toxicity testing, several hundred compounds are available and thoroughly characterized with traditional test systems. For reproductive toxicology, the number of compounds is smaller, but still sizable. For DNT, however, the very small number of known compounds leaves little choice. Moreover, none of the compounds is thoroughly characterized as to its mechanism of action. It is not known whether they can indeed be used as positive controls to assess perturbation of certain cellular processes. For example, it is not known, whether ethanol affects neural stem proliferation, migration or neurite growth. Its *in vivo* effect in humans may be perfectly explained by effects on differentiation of certain precursors. In assays modeling other stages or mechanisms of development, it could be used as a negative control compound. Another example is warfarin. This drug is developmentally toxic as it causes bleedings through anti-coagulating activity. It may serve as negative control in most cellular assays, as these are likely not to have a target for warfarin. Another disadvantage is that most known DNT compounds are non drug-like (mostly metals).

When using mechanistic tool compounds, the confidence to have a real positive compound within the context of the assay would be high, because the compound was chosen to exert a defined dysregulation. Transcriptional and other biomarkers could be related to known biochemical effects of the compounds. The separation between cytotoxicity and a specific effect may be good, as drug-like compounds with low general cytotoxicity can be chosen. Such compounds would be drugs or at least drug-like organic chemicals.

The major disadvantage is that it is unclear whether signaling disturbances are specific for DNT, and what the relevance of the compound is. Therefore, an intermediate solution would be to start with mechanistic tool compounds disrupting cellular processes that are known to be relevant for DNT, to better define assay characteristics. Then, one could proceed with a comparison of effects on cells that are not relevant for DNT (e.g. fibroblasts) to eliminate unspecific compounds. Finally, utilize some gold standard compounds that are known from other assays to trigger DNT, and that are expected to affect the biology relevant for the test system to be established, considering criteria defined in chapter 7.1 and 8.2.

8.2. Notes on technical and practical aspects in compound selection

8.2.1. Chemical properties of compounds

One consideration not to be neglected during the compound selection process are chemical properties of the compounds such as solubility and volatility. Cell culture systems are water-based, incubated at 37° C, usually in 20% O₂ and 5% CO₂ and are buffered to remain at physiological pH. Organic compounds that are not water-soluble may therefore cause problems since it cannot be guaranteed that once dissolved in water (even if solubilized in DMSO before) they will not precipitate or separate from the aqueous phase in other ways. Another problem often not considered are highly volatile compounds that may not stay in solution at 37°C, such that the exposure

concentration is uncertain. Examples are toluene, ethanol or hexane.

Also, there is an absolute requirement for a solvent control. Most ESC differentiation protocols are exquisitely sensitive to even slight changes in media composition, and the addition of, under other circumstances negligible amounts of a solvent such as DMSO, may impact the differentiation process. DMSO has been shown to trigger differentiation *in vitro* at low concentrations (105, 106). Accordingly, effects of addition of DMSO to the differentiation process should be evaluated.

To minimize the number of different solvent control cultures, it is advisable to only use compounds which are either soluble in DMSO or in cell culture medium during the initial phase of assay development.

8.2.2. Concentration-dependence

The essence of toxicology is that all its approaches, methods, systems and conclusions are strictly concentration-dependent. In other words, there might be a low-level biological response that gradually increases to the cytotoxic concentration comparable to the lethal dose in animal tests. Different concentrations of compounds might not only have quantitatively different effects, also the quality of the effect, the target and the mode of action may be entirely different at different concentrations.

8.2.3. Different modes of action at different concentrations

In toxicology, chemicals are often classified by their (a) target organ (e.g. nephro- or hepatotoxins) or (b) by their mode of action (e.g. AChE inhibitors). In pharmacology the 'mode of action' (MoA) approach is even more pronounced since drugs are usually developed for specific biological targets and the dose/concentration should only affect the magnitude of this specific effect without any qualitative shift. For the initial setup of a test system, compounds with distinct biological targets and MoA are useful to provoke specific effects.

There are three concentration-related general principles of using compounds in test development:

- compounds can be used for specific purposes at one or more concentrations, but not at every concentration,
- compounds can be used for different purposes at different concentrations,
- only the combination of compound + concentration allows a meaningful conclusion.

Although test compound lists are provided, these are only meaningful in certain concentration ranges. E.g. MeHg will be cytotoxic to every cell type at high concentrations, whereas specific DNT effects will only be observed at low concentrations.

9. PRACTICAL SELECTION OF COMPOUNDS

As already mentioned above, different types of compounds have to be selected for different assays and

Table 1. Compounds likely to cause DNT in humans

Compound	Human-relevant measurements ^{a)}	Comment	Ref.
Ethanol	4 g/d in mothers during pregnancy	fetal alcohol and solvent syndrome, associated with decreased IQ scores even at low consumption (3)	(116)
PCB	1.2 ng/ml serum at 42 months	neurobehavioral deficits in children prenatally exposed to PCB was found in several epidemiological studies (3)	(5, 117)
PBDE	0.5 to 3.3 µg/kg/day	ubiquitous flame retardant	(118)
Chlorpyrifos oxone	4.6 pg/g in cord blood	Active metabolite of chlorpyrifos	(119)
Dieldrin	no accurate quantifications	convulsant due to antagonism of the γ -aminobutyric acid-A (GABA(A)) receptor. Also endocrine disruptor (121). No metabolism required.	(120, 121)
Manganese	40 µg/l cord blood	impaired neurobehavioral development has been associated with high manganese concentrations in cord blood (122)	(122)
Arsenic	6.3 µg/l in venous whole blood samples	mental retardation, emotional disturbance (3)	(123)
Mercury	5 – 500 nM ^{b)} in brain	based on measurements and biomarkers after human poisoning (3)	(44)
Lead	0.5 – 1 µM	widespread subclinical neurobehavioral deficits confirmed in various epidemiological studies in children, maybe below concentrations that have been considered to be “safe” (3)	(25)
Valproic acid	over 800 mg/l (= 500-1000µM)	HDAC inhibitor; affects cell cycle, Wnt signaling and other processes; neural tube defects and reduced IQ	(124-126)

a) Human exposure measurements are often poorly documented, and relevant brain concentrations only scarcely available. For a rough orientation, different dose and concentration data are given as examples. More primary data and better kinetic modeling are necessary to obtain better estimates of target concentrations. If ranges are given, the lower number indicates the lower range of human sensitivity with respect to DNT, the higher number indicates typical concentrations that show adverse effects in most cases b) Calculated for methylmercury

even for different phases during the development of one specific assay (6). In general, all assays should be evaluated at some stage with the following 4 classes of compounds, to enable interpretation of the effects measured with the assay.

9.1. Known DNT compounds

Only a handful of substances is known to impact on human neurodevelopment, namely methylmercury, arsenic and lead compounds, ethanol, toluene, polychlorinated biphenyls (PCBs) (3). These compounds can be helpful during assay development. Due to epidemiological studies in humans, including careful measurements of target tissue concentrations, they can provide useful insights into the assay performance, and allow extrapolations from the *in vitro* assay to the *in vivo* situation (44). In addition to known human DNT compounds, substances that have been shown to impact neurodevelopment in animal systems can be used to broaden the possible spectrum of compounds. However, extrapolations to the human situations have to be made carefully. When using these known DNT compounds, it is of great importance to know the mode of action and to obtain information on whether the target or pathway affected by the compound is present in the system. For instance compounds that disrupt thyroid function or that damage the micro vessels are potentially causing developmental toxicity in animals (and man), but they may not be detected as toxic in an *in vitro* test system. Examples are summarized in Table 1. These “gold standard” compounds can provide information particularly on assay sensitivity, as the human relevant concentrations are known.

9.2. Negative controls

An important class of compounds often not considered, are negative control compounds. Such compounds are needed to evaluate the specificity of a given assay. One class of good negative compounds would be

compounds which are not expected to have an effect on the cellular system utilized when administered at high concentrations. These include e.g. sugars such as mannitol or sorbitol. Substances such as ascorbate and penicillin G, used as negative compounds in several reports should be avoided, as both impact on differentiation (107-109). A second class of possible negative control compounds includes compounds with a known mode of action and target. After having checked carefully for the absence of the target of the potential negative compound these compounds can serve as ideal negative controls. Such compounds could also include known DNT compounds like cyclopamine, depending on the type of assay.

The exact differentiation protocol conditions and all additives have to be considered carefully for the choice of negative compounds, particularly if bioactive molecules are added as part of the differentiation protocol, such as morphogens and cytokines. A list of possible negative control compounds is compiled in Table 2.

9.3. Pathway specific tool compounds

Due to the lack of many well characterized known DNT compounds, the first step when establishing an *in vitro* test system for developmental neurotoxicity is to use compounds targeting pathways known to be important for neurodevelopment. Such compounds can be used as endpoint-specific controls, as defined earlier (6). Again a special focus has to be on pathways that are relevant in the system used.

Cyclopamine is a good example for a compound that might be used in a particular assay as positive control, if the assay is depending on sonic hedgehog signaling, while in another assay it could serve as negative control. Cyclopamine is a naturally occurring alkaloid affecting neural development by disrupting patterning gradients at a very precise moment of gestation. In lambs, when ingested on the 14th day of gestation, this alkaloid induces severe

Table 2. Negative control compounds

Compound	Primary target	Comment
Saccharine		artificial sweetener
Sorbitol		artificial sweetener, osmotic diuretic agent, can open/cross the blood-brain barrier/blood-spinal cord barrier (127)
Mannitol		artificial sweetener, cell impermeant
Propranolol	beta ₁ - and beta ₂ -adrenergic receptor antagonist	
Glyphosate	assumed to block amino acid synthesis	broad-spectrum systemic herbicide, target thought to be absent in most <i>in vitro</i> systems
Diethylene glycol	liver and kidney	coolant, with low acute toxicity
Paracetamol	noncovalent interaction with COX-3	non-steroidal anti-inflammatory antipyretic drug (low anti-inflammatory), NO scavenger
Ibuprofen	COX	nonsteroidal anti-inflammatory drug, that might have neurotoxic side effects in rare cases (98)
Warfarin	vitamin K antagonist. Prevents gamma-carboxylation of clotting factors	target thought to be absent <i>in vitro</i>
Captopril	angiotensin-converting enzyme inhibitor	target thought to be absent in DNT systems
Dabigatran/rivaroxaban	Thrombin inhibitor	target thought to be absent <i>in vitro</i>
3-Methylpentane 2-Methylpentane	control for hexane	n-hexane isomer not metabolized to 2,5-hexanedione

Compounds higher up in the list are more likely to be true negatives. Negative controls depend on the test system used and on the concentrations selected. Relative measurements and tandem approaches may be required in some cases.

malformations such as cyclopia, cebocephaly, anophthalmia, or microphthalmia (110). However, when taken up earlier or later, no adverse effect can be detected. Other important pathways include Wnt, Notch, retinoid, MAPK/ERK and FGF signaling. Also, more general cellular functions, such as cytoskeleton rearrangements, might be useful targets for assays which assess cell migration or neurite outgrowth.

Other biological processes important for cell differentiation are epigenetic pathways, such as histone modification (e.g. acetyl-, methylation), DNA methylation or chromatin remodeling. Therefore, compounds interfering with these processes such as valproic acid, trichostatin A or 5-azacytidin might also be good pathway-specific tool compound (111). Some useful pathway specific tool compounds can be found in Table 3.

9.4. Generally cytotoxic compounds

The strength and uniqueness of *in vitro* test system for developmental neurotoxicity as discussed in this review is the identification of chemical effects on normal neural differentiation, and of disturbances that affect cellular morphology and function, but not overall survival. Identification of simple cytotoxicity can be achieved with easier and cheaper systems, and cytotoxic effects must be mostly regarded as artifacts for assays as discussed here (17, 53). Consequently, effects of cytotoxicity need to be distinguished from “real” prenatal neurotoxicity or developmental neurotoxicity. Therefore, general and unspecific cytotoxic compounds such as cytostatic drugs (e.g. 5-FU), detergents or inducers of apoptosis like staurosporin should be included in the initial compound list. As cytotoxicity can affect the transcriptome (e.g. up-regulation of apoptosis-related genes like *CASP3*, or of other stress markers such as heme oxygenase), genetic markers triggered by unspecific cytotoxicants may be used later on to discriminate between pure cytotoxicity and specific developmental neurotoxic effects of compounds. Also, selection of this class of compounds requires great care and adaptation to the respective assay and purpose. In many cases, compounds killing cells by excessive

production of reactive oxygen species or by blocking energy regeneration may be considered non-specific. However, there are examples where oxidative stress controls neurodifferentiation or specifically affects a (dopaminergic) subpopulation of immature human neurons (112-113). Also, apparently unspecific mitochondrial toxicants show specific effects on defined neuronal subtypes, and mitochondrial respiratory chain inhibitors affect e.g cardiac differentiation (114-115).

Generally-cytotoxic compounds with diverse mode of action are compiled in Table 4.

10. PERSPECTIVE

As faster and cheaper approaches are required to test drugs and other chemicals for their neurodevelopmental hazard, there is an urgency to develop new alternative assays to circumvent the bottle neck of the established animal-based test systems. As cellular *in vitro* test systems, including models based on hESC and their progeny, promise to provide biological relevance to human toxicity testing, it is crucial to carefully design, characterize and evaluate such assays. The most straight-forward approach is the use of chemicals with known activities in animals and man to examine the quality and predictivity of such new *in vitro* tests. Work on the selection of such chemicals has shown that the available *in vivo* information is often limited, and in most cases, it is not sufficient at all to define expected modes of action *in vitro*. Thus, it appears as if more *in vivo* work will be required to characterize compounds required for the development of *in vitro* methods. In the absence of such information, an alternative scientific solution may be the extensive characterization of compounds for their biological activity in a battery of quality controlled *in vitro* assays. This information may then be used (a) to define focused animal experiments to test the predictions from the test battery and to obtain proof of the relevance of the selected tool compounds, and (b) to evaluate the predictivity of further assays with the help of such a collection of chemicals, characterized extensively for their *in vitro* modes of action.

Compounds for the development of *in vitro* DNT assays

Table 3. Pathway specific compounds

Chemical	MoA/Target	Comment
Pathway inhibitors		
DAPT	Notch	impact on NPC differentiation (128)
Cyclopamine	hedgehog (Hh) signaling	Impact on brain patterning (129)
Rapamycin	mTOR	mTOR crucial for directional growth of axons, development of the neuronal dendritic tree, formation of proper synaptic connections, during neuronal development (130)
LiCl, SB-216763	Wnt signaling/ GSK3-inhibition	improves neurogenesis (131)
Valproic acid	HDAC inhibitor	anticonvulsant (fetal antiepileptic drug syndrome) (98), suspected to cause neural tube defects
U0126, PD98059	MEK1/2	inhibits neurite outgrowth (53)
Wortmannin	PI3K	inhibits neurite outgrowth (132)
Bisindolylmaleimide	PKC	inhibits neurite outgrowth (53)
Cyclosporine	Cyclophilin	inhibits calcium signaling
Simvastatin	Inhibitor of HMG-CoA reductase	Statin, affects lipid rafts and small G proteins
Forskolin	adenylate cyclase activator	Activation of protein kinase A and CREB signaling
Y-27632	ROCK inhibitor	Anti-apoptotic, increases hESC single cell survival
SAHA	HDAC inhibitor	Modulates differentiation
5-azacytidine	DNA methylation	cytidine analogue, may stop cell proliferation
Imatinib (Glivec)	PDGF-R, c-kit, Src	inhibitor of ABL kinase, c-Kit, PDGFR
Gefitinib (Iressa)	EGF-R	inhibitor of the receptor-associated tyrosine kinase
Hormonally active		
T3/T4 (thyroxine)	thyroid hormone receptor	thyroid hormone
EGF	EGF receptor	growth factor for neural precursor cells
Estrogen	estrogen receptors	enhances adult neurogenesis (133)
Dexamethasone	anti-inflammatory, immunosuppressant	member of the glucocorticoid class of steroid drugs
RU38468 (Mifepristone)	progesterone and glucocorticoid receptor antagonist	synthetic steroid
BMP4	morphogen	inhibition leads to neural differentiation
Tamoxifen	estrogen receptors	competitive estrogen receptor antagonist
Rosiglitazone	PPARgamma	Insulin sensitizer
AMD3100	Chemokine signaling (CXCR4)	migration of neural precursor cells

Table 4. Generally cytotoxic compounds

Compound	MoA/target	Comment
Staurosporine	apoptosis	broad spectrum kinase inhibitor
Buthionine sulfoximine	oxidative stress	inhibits γ -glutamylcysteine synthetase
MG132	proteasome block	blocks NF-kB activation
Digoxin	Na/K ATPase	disturbed ion homeostasis
Dinitrophenol	mitochondrial uncoupler	causes energy failure
5-FU	base analogon antimetabolite	inhibits DNA synthesis, S-phase specific mitosis block
Menadione	redoxcycler	causes oxidative stress
Okadaic acid	protein phosphatase inhibitor	cytoskeleton/signaling chaos
Doxorubicin	DNA intercalation	replication stop/oxidative stress
H-33342	DNA intercalation	RNA synthesis block
Etoposide	inhibits topoisomerase II	DNA strand breaks; apoptosis
Camptothecin	inhibits topoisomerase I	Apoptosis
Deoxyglucose	block of glycolysis	ATP depletion
Ochratoxin	kidney specific (non CNS)	inhibition of protein synthesis
Colchicine	inhibits microtubule assembly	cytoskeleton/axonal transport/cell cycle disruption
Taxol	stabilizes microtubules	axonal transport/cell cycle disruption
Thapsigargin	blocks sarco/endoplasmic reticulum Ca^{2+} ATPases	raises cytosolic calcium, causes ER stress

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12. REFERENCES

1. Makris, S. L., K. Raffaele, S. Allen, W. J. Bowers, U. Hass, E. Alleva, G. Calamandrei, L. Sheets, P. Amcoff, N.

Delrue and K. M. Crofton: A retrospective performance assessment of the developmental neurotoxicity study in support of OECD test guideline 426. *Environ Health Perspect* 117, 17-25 (2009)

2. Middaugh, L. D., D. Dow-Edwards, A. A. Li, J. D. Sandler, J. Seed, L. P. Sheets, D. L. Shuey, W. Slikker, Jr., W. P. Weisenburger, L. D. Wise and M. R. Selwyn: Neurobehavioral assessment: a survey of use and value in safety assessment studies. *Toxicol Sci* 76, 250-61 (2003)

3. Grandjean, P. and P. J. Landrigan: Developmental neurotoxicity of industrial chemicals. *The Lancet* 368, 2167-2178 (2006)

4. Boulet, S. L., C. A. Boyle and L. A. Schieve: Health Care Use and Health and Functional Impact of Developmental Disabilities Among US Children, 1997-2005. *Arch Pediatr Adolesc Med* 163, 19-26 (2009)
5. Walkowiak, J., J. A. Wiener, A. Fastabend, B. Heinzow, U. Kramer, E. Schmidt, H. J. Steingruber, S. Wundram and G. Winneke: Environmental exposure to polychlorinated biphenyls and quality of the home environment: effects on psychodevelopment in early childhood. *Lancet* 358, 1602-7 (2001)
6. Crofton, K. M., W. R. Mundy, P. J. Lein, A. Bal-Price, S. Coecke, A. E. Seiler, H. Knaut, L. Buzanska and A. Goldberg: Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. *Altex* 28, 9-15 (2011)
7. van Thriel, C., R. Westernick, C. Best, A. Bale, P. Lein and M. Leist: Translating neurobehavioural endpoints of developmental neurotoxicity tests into *in vitro* assays and readouts. *Neurotoxicology* in press (2011)
8. Leist, M., T. Hartung and P. Nicotera: The dawning of a new age of toxicology. *Altex* 25, 103-14 (2008)
9. Leist, M., S. Bremer, P. Brundin, J. Hescheler, A. Kirkeby, K. H. Krause, P. Poerzgen, M. Puceat, M. Schmidt, A. Schratzenholz, N. B. Zak and H. Hentze: The biological and ethical basis of the use of human embryonic stem cells for *in vitro* test systems or cell therapy. *Altex* 25, 163-90 (2008)
10. Leist, M., S. Kadereit and S. Schildknecht: Food for thought... on the real success of 3R approaches. *Altex* 25, 17-32 (2008)
11. Barberi, T., P. Klivenyi, N. Y. Calingasan, H. Lee, H. Kawamata, K. Loonam, A. L. Perrier, J. Bruses, M. E. Rubio, N. Topf, V. Tabar, N. L. Harrison, M. F. Beal, M. A. Moore and L. Studer: Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol* 21, 1200-7 (2003)
12. Abranches, E., M. Silva, L. Pradier, H. Schulz, O. Hummel, D. Henrique and E. Bekman: Neural Differentiation of Embryonic Stem Cells *<italic>In vitro</italic>*: A Road Map to Neurogenesis in the Embryo. *PLoS One* 4, e6286 (2009)
13. Conti, L. and E. Cattaneo: Neural stem cell systems: physiological players or *in vitro* entities? *Nat Rev Neurosci* 11, 176-187 (2010)
14. Elkabetz, Y. and L. Studer: Human ESC-derived neural rosettes and neural stem cell progression. *Cold Spring Harb Symp Quant Biol* 73, 377-87 (2008)
15. Fasano, C. A., S. M. Chambers, G. Lee, M. J. Tomishima and L. Studer: Efficient Derivation of Functional Floor Plate Tissue from Human Embryonic Stem Cells. *Cell Stem Cell* 6, 336-347 (2010)
16. Colleoni, S., C. BGalli, J. Gaspar, K. Meganathan, S. Jagtap, J. Hescheler, A. Sachinidis and G. Lazzari: Development of a neural teratogenicity test based on human embryonic stem cells: response to retinoic acid exposure. *Toxicological Sciences* in press (2011)
17. Leist, M., L. Efremova and C. Karreman: Food for thought ... considerations and guidelines for basic test method descriptions in toxicology. *Altex* 27, 309-17 (2010)
18. Rao, M.: Stem and precursor cells in the nervous system. *J Neurotrauma* 21, 415-27 (2004)
19. Wilson, L. and M. Maden: The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Dev Biol* 282, 1-13 (2005)
20. Evans, M. J. and M. H. Kaufman: Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-6 (1981)
21. Martin, G. R.: Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78, 7634-8 (1981)
22. Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones: Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-7 (1998)
23. Lee, G., E. P. Papapetrou, H. Kim, S. M. Chambers, M. J. Tomishima, C. A. Fasano, Y. M. Ganat, J. Menon, F. Shimizu, A. Viale, V. Tabar, M. Sadelain and L. Studer: Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 461, 402-6 (2009)
24. Perrier, A. L., V. Tabar, T. Barberi, M. E. Rubio, J. Bruses, N. Topf, N. L. Harrison and L. Studer: Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 101, 12543-8 (2004)
25. Zimmer, B., P. B. Kuegler, B. Baudis, A. Genewsky, V. Tanavde, W. Koh, B. Tan, T. Waldmann, S. Kadereit and M. Leist: Coordinated waves of gene expression during neuronal differentiation of embryonic stem cells as basis for novel approaches to developmental neurotoxicity testing. *Cell Death Differ* 18, 383-95 (2011)
26. Jagtap, S., K. Meganathan, J. Gaspar, V. Wagh, J. Winkler, J. Hescheler and A. Sachinidis: Cytosine arabinoside induces ectoderm and inhibits mesoderm expression in human embryonic stem cells during multilineage differentiation. *British Journal of Pharmacology* 162, 1743-1756 (2011)
27. Stummann, T. C., L. Hareng and S. Bremer: Hazard assessment of methylmercury toxicity to neuronal induction

in embryogenesis using human embryonic stem cells. *Toxicology* 257, 117-126 (2009)

28. Theunissen, P. T., J. L. A. Pennings, J. F. Robinson, S. M. H. Claessen, J. C. S. Kleinjans and A. H. Piersma: Time-Response Evaluation by Transcriptomics of Methylmercury Effects on Neural Differentiation of Murine Embryonic Stem Cells. *Toxicological Sciences* 122, 437-447 (2011)

29. Zeng, X., J. Chen, X. Deng, Y. Liu, M. S. Rao, J. L. Cadet and W. J. Freed: An *in vitro* model of human dopaminergic neurons derived from embryonic stem cells: MPP⁺ toxicity and GDNF neuroprotection. *Neuropsychopharmacology* 31, 2708-15 (2006)

30. Belmonte, M. and T. Bourgeron: Fragile X syndrome and autism at the intersection of genetic and neural networks. *Nature Neuroscience* 9, 1221-1225 (2006)

31. Berti, V., B. Nacmias, S. Bagnoli and S. Sorbi: Alzheimer's disease: genetic basis and amyloid imaging as endophenotype. *Q J Nucl Med Mol Imaging* 55, 225-36 (2011)

32. Gottesman, II and T. D. Gould: The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* 160, 636-45 (2003)

33. Gould, T. D. and Gottesman, II: Psychiatric endophenotypes and the development of valid animal models. *Genes Brain Behav* 5, 113-9 (2006)

34. Hasler, G. and G. Northoff: Discovering imaging endophenotypes for major depression. *Mol Psychiatry* 16, 604-19 (2011)

35. Hammer, T. B., B. Oranje, B. Fagerlund, H. Bro and B. Y. Glenthøj: Stability of prepulse inhibition and habituation of the startle reflex in schizophrenia: a 6-year follow-up study of initially antipsychotic-naïve, first-episode schizophrenia patients. *Int J Neuropsychopharmacol* 14, 913-25 (2011)

36. Roussos, P., S. G. Giakoumaki, E. Adamaki and P. Bitsios: The influence of schizophrenia-related neuregulin-1 polymorphisms on sensorimotor gating in healthy males. *Biol Psychiatry* 69, 479-86 (2011)

37. Penagarikano, O., B. S. Abrahams, E. I. Herman, K. D. Winden, A. Gdalyahu, H. Dong, L. I. Sonnenblick, R. Gruver, J. Almajano, A. Bragin, P. Golshani, J. T. Trachtenberg, E. Peles and D. H. Geschwind: Absence of CNTNAP2 Leads to Epilepsy, Neuronal Migration Abnormalities, and Core Autism-Related Deficits. *Cell* 147, 235-46 (2011)

38. Hartung, T. and M. McBride: Food for Thought ... on mapping the human toxome. *Altex* 28, 83-93 (2011)

39. Andersen, M. E., H. J. Clewell, P. L. Carmichael and K. Boekelheide: Can case study approaches speed

implementation of the NRC report: "Toxicity Testing in the 21st Century: A Vision and a Strategy?". *Altex* 28, 175-82 (2011)

40. Committee on Toxicity Testing and Assessment of Environmental Agents, N. R. C.: Toxicity Testing in the 21st Century: A Vision and a Strategy. *The National Academies Press* (2007)

41. Ankley, G. T., R. S. Bennett, R. J. Erickson, D. J. Hoff, M. W. Hornung, R. D. Johnson, D. R. Mount, J. W. Nichols, C. L. Russom, P. K. Schmieder, J. A. Serrano, J. E. Tietge and D. L. Villeneuve: Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29, 730-41 (2010)

42. Watanabe, K. H., M. E. Andersen, N. Basu, M. J. Carvan, 3rd, K. M. Crofton, K. A. King, C. Sunol, E. Tiffany-Castiglioni and I. R. Schultz: Defining and modeling known adverse outcome pathways: Domoic acid and neuronal signaling as a case study. *Environ Toxicol Chem* 30, 9-21 (2011)

43. Kuegler, P. B., B. Zimmer, T. Waldmann, B. Baudis, S. Ilmjarv, J. Hescheler, P. Gaughwin, P. Brundin, W. Mundy, A. K. Bal-Price, A. Schrattenholz, K. H. Krause, C. van Thriel, M. S. Rao, S. Kadereit and M. Leist: Markers of murine embryonic and neural stem cells, neurons and astrocytes: reference points for developmental neurotoxicity testing. *Altex* 27, 17-42 (2010)

44. Zimmer, B., S. Schildknecht, P. B. Kuegler, V. Tanavde, S. Kadereit and M. Leist: Sensitivity of dopaminergic neuron differentiation from stem cells to chronic low-dose methylmercury exposure. *Toxicol Sci* 121, 357-67 (2011)

45. Faustman, E. M., R. A. Ponce, Y. C. Ou, M. A. Mendoza, T. Lewandowski and T. Kavanagh: Investigations of methylmercury-induced alterations in neurogenesis. *Environ Health Perspect* 110 Suppl 5, 859-64 (2002)

46. Johansson, C., A. F. Castoldi, N. Onishchenko, L. Manzo, M. Vahter and S. Ceccatelli: Neurobehavioural and molecular changes induced by methylmercury exposure during development. *Neurotox Res* 11, 241-60 (2007)

47. Radio, N. M. and W. R. Mundy: Developmental neurotoxicity testing *in vitro*: models for assessing chemical effects on neurite outgrowth. *Neurotoxicology* 29, 361-76 (2008)

48. Parran, D. K., S. Barone, Jr. and W. R. Mundy: Methylmercury decreases NGF-induced TrkA autophosphorylation and neurite outgrowth in PC12 cells. *Brain Res Dev Brain Res* 141, 71-81. (2003)

49. Radio, N. M., J. M. Breier, T. J. Shafer and W. R. Mundy: Assessment of chemical effects on neurite outgrowth in PC12 cells using high content screening. *Toxicol Sci* 105, 106-18 (2008)

50. Moors, M., T. D. Rockel, J. Abel, J. E. Cline, K. Gassmann, T. Schreiber, J. Schuwald, N. Weinmann and E. Fritsche: Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ Health Perspect* 117, 1131-8 (2009)
51. Harrill, J. A., T. M. Freudenrich, D. W. Machacek, S. L. Stice and W. R. Mundy: Quantitative assessment of neurite outgrowth in human embryonic stem cell-derived hN2(TM) cells using automated high-content image analysis. *NeuroToxicology* 31, 277-290 (2010)
52. Radio, N. M., T. M. Freudenrich, B. L. Robinette, K. M. Crofton and W. R. Mundy: Comparison of PC12 and cerebellar granule cell cultures for evaluating neurite outgrowth using high content analysis. *Neurotoxicol Teratol* 32, 25-35 (2010)
53. Stiegler, N. V., A. K. Krug, F. Matt and M. Leist: Assessment of chemical-induced impairment of human neurite outgrowth by multiparametric live cell imaging in high-density cultures. *Toxicol Sci* 121, 73-87 (2011)
54. Barde, Y.-A.: Trophic factors and neuronal survival. *Neuron* 2, 1525-1534 (1989)
55. Farlie, P. G., R. Dringen, S. M. Rees, G. Kannourakis and O. Bernard: bcl-2 transgene expression can protect neurons against developmental and induced cell death. *Proceedings of the National Academy of Sciences* 92, 4397-4401 (1995)
56. Cimadamore, F., C. L. Curchoe, N. Alderson, F. Scott, G. Salvesen and A. V. Terskikh: Nicotinamide rescues human embryonic stem cell-derived neuroectoderm from parthanatic cell death. *Stem Cells* 27, 1772-81 (2009)
57. Ciaroni, S., T. Cecchini, P. Ferri, P. Ambrogini, R. Cuppini, M. Riccio, G. Lombardelli, S. Papa and P. Del Grande: Impairment of neural precursor proliferation increases survival of cell progeny in the adult rat dentate gyrus. *Mechanisms of Ageing and Development* 123, 1341-1352 (2002)
58. Blomgren, K., M. Leist and L. Groc: Pathological apoptosis in the developing brain. *Apoptosis* 12, 993-1010 (2007)
59. Valiente, M. and O. Marin: Neuronal migration mechanisms in development and disease. *Curr Opin Neurobiol* 20, 68-78 (2010)
60. Ruhrberg, C. and Q. Schwarz: In the beginning: Generating neural crest cell diversity. *Cell Adh Migr* 4, 622-30 (2010)
61. Nobes, C. D. and A. Hall: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53-62 (1995)
62. Becchetti, A. and A. Arcangeli: Integrins and ion channels in cell migration: implications for neuronal development, wound healing and metastatic spread. *Adv Exp Med Biol* 674, 107-23 (2010)
63. Kurosaka, S. and A. Kashina: Cell biology of embryonic migration. *Birth Defects Res C Embryo Today* 84, 102-22 (2008)
64. Patapoutian, A. and L. F. Reichardt: Roles of Wnt proteins in neural development and maintenance. *Current Opinion in Neurobiology* 10, 392-399 (2000)
65. Zhang, S.-C., M. Wernig, I. D. Duncan, O. Brustle and J. A. Thomson: *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotech* 19, 1129-1133 (2001)
66. Munoz-Sanjuan, I. and A. H. Brivanlou: Neural induction, the default model and embryonic stem cells. *Nat Rev Neurosci* 3, 271-280 (2002)
67. Ding, S., T. Y. H. Wu, A. Brinker, E. C. Peters, W. Hur, N. S. Gray and P. G. Schultz: Synthetic small molecules that control stem cell fate. *Proceedings of the National Academy of Sciences* 100, 7632-7637 (2003)
68. Louvi, A. and S. Artavanis-Tsakonas: Notch signalling in vertebrate neural development. *Nat Rev Neurosci* 7, 93-102 (2006)
69. Chambers, S. M., C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain and L. Studer: Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotech* 27, 275-280 (2009)
70. Krencik, R. and S. C. Zhang: Stem cell neural differentiation: A model for chemical biology. *Curr Opin Chem Biol* 10, 592-7 (2006)
71. Cezar, G. G., J. A. Quam, A. M. Smith, G. J. Rosa, M. S. Piekarczyk, J. F. Brown, F. H. Gage and A. R. Muotri: Identification of small molecules from human embryonic stem cells using metabolomics. *Stem Cells Dev* 16, 869-82 (2007)
72. Vo, N. K., X. A. Cambronne and R. H. Goodman: MicroRNA pathways in neural development and plasticity. *Current Opinion in Neurobiology* 20, 457-465 (2010)
73. West, P. R., A. M. Weir, A. M. Smith, E. L. R. Donley and G. G. Cezar: Predicting human developmental toxicity of pharmaceuticals using human embryonic stem cells and metabolomics. *Toxicology and Applied Pharmacology* 247, 18-27 (2010)
74. Gossrau, G., J. Thiele, R. Konang, T. Schmandt and O. Brustle: Bone morphogenetic protein-mediated modulation of lineage diversification during neural differentiation of embryonic stem cells. *Stem Cells* 25, 939-49 (2007)

75. Clancy, B., B. L. Finlay, R. B. Darlington and K. J. Anand: Extrapolating brain development from experimental species to humans. *Neurotoxicology* 28, 931-7 (2007)
76. Falsig, J., P. Porzgen, J. Lotharius and M. Leist: Specific modulation of astrocyte inflammation by inhibition of mixed lineage kinases with CEP-1347. *J Immunol.* 173, 2762-70. (2004)
77. Falsig, J., P. Porzgen, S. Lund, A. Schrattenholz and M. Leist: The inflammatory transcriptome of reactive murine astrocytes and implications for their innate immune function. *J Neurochem.* 96, 893-907. Epub 2006 Jan 9. (2006)
78. Lund, S., K. V. Christensen, M. Hedtjarn, A. L. Mortensen, H. Hagberg, J. Falsig, H. Hasseldam, A. Schrattenholz, P. Porzgen and M. Leist: The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and *in vivo* conditions. *J Neuroimmunol* 180, 71-87 (2006)
79. Kuegler, P., B. Baumann, B. Zimmer, S. Keller, A. Marx, S. Kadereit and M. Leist: GFAP-independent inflammatory competence and trophic functions of astrocytes generated from murine embryonic stem cells. *Glia* (in press)
80. Powell, E. M., S. Meiners, N. A. DiProspero and H. M. Geller: Mechanisms of astrocyte-directed neurite guidance. *Cell Tissue Res* 290, 385-93 (1997)
81. Tarone, G., E. Hirsch, M. Brancaccio, M. De Acetis, L. Barberis, F. Balzac, S. F. Retta, C. Botta, F. Altruda and L. Silengo: Integrin function and regulation in development. *Int J Dev Biol* 44, 725-31 (2000)
82. Kiryushko, D., V. Berezin and E. Bock: Regulators of neurite outgrowth: role of cell adhesion molecules. *Ann N Y Acad Sci* 1014, 140-54 (2004)
83. Yoshimura, T., N. Arimura and K. Kaibuchi: Signaling networks in neuronal polarization. *J Neurosci* 26, 10626-30 (2006)
84. Drachman, D. A.: Do we have brain to spare? *Neurology* 64, 2004-5 (2005)
85. Munno, D. W. and N. I. Syed: Synaptogenesis in the CNS: an odyssey from wiring together to firing together. *J Physiol* 552, 1-11 (2003)
86. Garner, C. C., R. G. Zhai, E. D. Gundelfinger and N. E. Ziv: Molecular mechanisms of CNS synaptogenesis. *Trends Neurosci* 25, 243-51 (2002)
87. de Jong, E., A. M. Doedee, M. A. Reis-Fernandes, H. Nau and A. H. Piersma: Potency ranking of valproic acid analogues as to inhibition of cardiac differentiation of embryonic stem cells in comparison to their *in vivo* embryotoxicity. *Reprod Toxicol* 31, 375-82 (2011)
88. Nolen, B. J., N. Tomasevic, A. Russell, D. W. Pierce, Z. Jia, C. D. McCormick, J. Hartman, R. Sakowicz and T. D. Pollard: Characterization of two classes of small molecule inhibitors of Arp2/3 complex. *Nature* 460, 1031-1034 (2009)
89. Monastyrska, I., C. He, J. Geng, A. D. Hoppe, Z. Li and D. J. Klionsky: Arp2 Links Autophagic Machinery with the Actin Cytoskeleton. *Mol. Biol. Cell* 19, 1962-1975 (2008)
90. Zhu, S., K. T. Mc Henry, W. S. Lane and G. Fenteany: A Chemical Inhibitor Reveals the Role of Raf Kinase Inhibitor Protein in Cell Migration. *Chemistry & biology* 12, 981-991 (2005)
91. Mc Henry, K. T., R. Montesano, S. Zhu, A. B. Beshir, H.-H. Tang, K. C. Yeung and G. Fenteany: Raf kinase inhibitor protein positively regulates cell-substratum adhesion while negatively regulating cell-cell adhesion. *Journal of Cellular Biochemistry* 103, 972-985 (2008)
92. Schildknecht, S., R. Pape, N. Muller, M. Robotta, A. Marquardt, A. Burkle, M. Drescher and M. Leist: Neuroprotection by minocycline caused by direct and specific scavenging of peroxynitrite. *J Biol Chem* 286, 4991-5002 (2011)
93. Schildknecht, S., D. Poltl, D. M. Nagel, F. Matt, D. Scholz, J. Lotharius, N. Schmieg, A. Salvo-Vargas and M. Leist: Requirement of a dopaminergic neuronal phenotype for toxicity of low concentrations of 1-methyl-4-phenylpyridinium to human cells. *Toxicol Appl Pharmacol* 241, 23-35 (2009)
94. Apati, A., T. I. Orban, N. Varga, A. Nemeth, A. Schamberger, V. Krizsik, B. Erdelyi-Belle, L. Homolya, G. Varady, R. Padanyi, E. Karaszi, E. W. Kemna, K. Nemet and B. Sarkadi: High level functional expression of the ABCG2 multidrug transporter in undifferentiated human embryonic stem cells. *Biochim Biophys Acta* 1778, 2700-9 (2008)
95. Kalgutkar, A. S. and H. T. Nguyen: Identification of an N-methyl-4-phenylpyridinium-like metabolite of the antidiarrheal agent loperamide in human liver microsomes: underlying reason(s) for the lack of neurotoxicity despite the bioactivation event. *Drug Metab Dispos* 32, 943-52 (2004)
96. Moser, V. C., P. M. Phillips, K. L. McDaniel and R. C. MacPhail: Behavioral evaluation of the neurotoxicity produced by dichloroacetic acid in rats. *Neurotoxicol Teratol* 21, 719-31 (1999)
97. Stehr, C. M., T. L. Linbo, J. P. Incardona and N. L. Scholz: The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. *Toxicol Sci* 92, 270-8 (2006)
98. Spencer, P., H. Schaumburg and A. Ludolph: Experimental and Clinical Neurotoxicology. In: Oxford University Press, New York (2000)

99. Sjöström, M., A. Kolman, C. Clemenson and R. Clothier: Estimation of human blood LC50 values for use in modeling of *in vitro-in vivo* data of the ACuteTox project. *Toxicology in vitro* 22, 1405-1411 (2008)
100. Bondesson, I., B. Ekwall, S. Hellberg, L. Romert, K. Stenberg and E. Walum: MEIC--a new international multicenter project to evaluate the relevance to human toxicity of *in vitro* cytotoxicity tests. *Cell Biol Toxicol* 5, 331-47 (1989)
101. Vinken, M., T. Doktorova, H. Ellinger-Ziegelbauer, H. J. Ahr, E. Lock, P. Carmichael, E. Roggen, J. van Delft, J. Kleijnans, J. Castell, R. Bort, T. Donato, M. Ryan, R. Corvi, H. Keun, T. Ebbels, T. Athersuch, S. A. Sansone, P. Rocca-Serra, R. Stierum, P. Jennings, W. Pfäller, H. Gmuender, T. Vanhaecke and V. Rogiers: The carcinoGENOMICS project: critical selection of model compounds for the development of omics-based *in vitro* carcinogenicity screening assays. *Mutat Res.* 659, 202-10. Epub 2008 Apr 26. (2008)
102. Pazos, P., C. Pellizzer, T. C. Stummann, L. Hareng and S. Bremer: The test chemical selection procedure of the European Centre for the Validation of Alternative Methods for the EU Project ReProTect. *Reprod Toxicol* 30, 161-99 (2010)
103. Chandler, K. J., M. Barrier, S. Jeffay, H. P. Nichols, N. C. Kleinstreuer, A. V. Singh, D. M. Reif, N. S. Sipes, R. S. Judson, D. J. Dix, R. Kavlock, E. S. Hunter, 3rd and T. B. Knudsen: Evaluation of 309 environmental chemicals using a mouse embryonic stem cell adherent cell differentiation and cytotoxicity assay. *PLoS One* 6, e18540 (2011)
104. Coecke, S., A. M. Goldberg, S. Allen, L. Buzanska, G. Calamandrei, K. Crofton, L. Hareng, T. Hartung, H. Knaut, P. Honnegger, M. Jacobs, P. Lein, A. Li, W. Mundy, D. Owen, S. Schneider, E. Silbergeld, T. Reum, T. Trnovec, F. Monnet-Tschudi and A. Bal-Price: Workgroup report: incorporating *in vitro* alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. *Environ Health Perspect* 115, 924-31 (2007)
105. Lyman, G. H., H. D. Preisler and D. Papahadjopoulos: Membrane action of DMSO and other chemical inducers of Friend leukaemic cell differentiation. *Nature* 262, 360-363 (1976)
106. Wang, H. and R. E. Scott: Inhibition of distinct steps in the adipocyte differentiation pathway in 3T3 T mesenchymal stem cells by dimethyl sulphoxide (DMSO). *Cell Prolif* 26, 55-66 (1993)
107. Takahashi, T., B. Lord, P. C. Schulze, R. M. Fryer, S. S. Sarang, S. R. Gullans and R. T. Lee: Ascorbic Acid Enhances Differentiation of Embryonic Stem Cells Into Cardiac Myocytes. *Circulation* 107, 1912-1916 (2003)
108. Uyanıkgil, Y., M. Baka, M. Yurtseven and M. Turgut: The effect of experimental epilepsy induced by penicillin administration during pregnancy on nestin expression in the immature rat cerebellum. *Child's Nervous System* 20, 176-182 (2004)
109. Haramoto, M., H. Tatemoto and N. Muto: Essential Role of Ascorbic Acid in Neural Differentiation and Development: High Levels of Ascorbic Acid 2-Glucoside Effectively Enhance Nerve Growth Factor-Induced Neurite Formation and Elongation in PC12 Cells *J. Health Sci.* 54, 43-49 (2008)
110. Keeler, R. F.: Cyclopamine and related steroidal alkaloid teratogens: their occurrence, structural relationship, and biologic effects. *Lipids* 13, 708-15 (1978)
111. Jung, J. W., S. Lee, M. S. Seo, S. B. Park, A. Kurtz, S. K. Kang and K. S. Kang: Histone deacetylase controls adult stem cell aging by balancing the expression of polycomb genes and jumonji domain containing 3. *Cell Mol Life Sci* 67, 1165-76 (2010)
112. Yan, Y., P. Sabharwal, M. Rao and S. Sockanathan: The Antioxidant Enzyme Prdx1 Controls Neuronal Differentiation by Thiol-Redox-Dependent Activation of GDE2. *Cell* 138, 1209-1221 (2009)
113. Hansson, O., R. F. Castilho, G. S. Kaminski Schierle, J. Karlsson, P. Nicotera, M. Leist and P. Brundin: Additive effects of caspase inhibitor and lazardol on the survival of transplanted rat and human embryonic dopamine neurons. *Exp Neurol* 164, 102-11 (2000)
114. Sherer, T. B., J. R. Richardson, C. M. Testa, B. B. Seo, A. V. Panov, T. Yagi, A. Matsuno-Yagi, G. W. Miller and J. T. Greenamyre: Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. *J Neurochem* 100, 1469-79 (2007)
115. San Martin, N., A. M. Cervera, C. Cordova, D. Covarello, K. J. McCreath and B. G. Galvez: Mitochondria determine the differentiation potential of cardiac mesoangioblasts. *Stem Cells* 29, 1064-74 (2011)
116. Fried, P. A., B. Watkinson and R. Gray: A follow-up study of attentional behavior in 6-year-old children exposed prenatally to marijuana, cigarettes, and alcohol. *Neurotoxicol Teratol* 14, 299-311 (1992)
117. Roegge, C. S., B. W. Seo, K. M. Crofton and S. L. Schantz: Gestational-lactational exposure to Aroclor 1254 impairs radial-arm maze performance in male rats. *Toxicol Sci* 57, 121-30 (2000)
118. Costa, L. G. and G. Giordano: Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology* 28, 1047-67 (2007)
119. Rauh, V., S. Arunajadai, M. Horton, F. Perera, L. Hoepner, D. B. Barr and R. Whyatt: Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. *Environ Health Perspect* 119, 1196-201 (2011)

120. Weisskopf, M. G., P. Knekt, E. J. O'Reilly, J. Lyytinen, A. Reunanen, F. Laden, L. Altshul and A. Ascherio: Persistent organochlorine pesticides in serum and risk of Parkinson disease. *Neurology* 74, 1055-61 (2010)
121. Briz, V., J. M. Molina-Molina, S. Sanchez-Redondo, M. F. Fernandez, J. O. Grimalt, N. Olea, E. Rodriguez-Farre and C. Sunol: Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan, and lindane in primary neuronal cultures. *Toxicol Sci* 120, 413-27 (2011)
122. Takser, L., D. Mergler, G. Hellier, J. Sahuquillo and G. Huel: Manganese, Monoamine Metabolite Levels at Birth, and Child Psychomotor Development. *NeuroToxicology* 24, 667-674 (2003)
123. Parvez, F., G. A. Wasserman, P. Factor-Litvak, X. Liu, V. Slavkovich, A. B. Siddique, R. Sultana, R. Sultana, T. Islam, D. Levy, J. L. Mey, A. van Geen, K. Khan, J. Kline, H. Ahsan and J. H. Graziano: Arsenic Exposure and Motor Function among Children in Bangladesh. *Environ Health Perspect* (2011)
124. Tomson, T. and D. Battino: Teratogenic effects of antiepileptic medications. *Neurol Clin* 27, 993-1002 (2009)
125. Werler, M. M., K. A. Ahrens, J. L. Bosco, A. A. Mitchell, M. T. Anderka, S. M. Gilboa and L. B. Holmes: Use of antiepileptic medications in pregnancy in relation to risks of birth defects. *Ann Epidemiol* 21, 842-50 (2011)
126. Meador, K. J., G. A. Baker, N. Browning, J. Clayton-Smith, D. T. Combs-Cantrell, M. Cohen, L. A. Kalayjian, A. Kanner, J. D. Liporace, P. B. Pennell, M. Privitera and D. W. Loring: Cognitive function at 3 years of age after fetal exposure to antiepileptic drugs. *N Engl J Med* 360, 1597-605 (2009)
127. Prockop, L. D., K. A. Naidu, J. E. Binard and J. Ransohoff: Selective permeability of [3H]-D-mannitol and [14C]-carboxyl-inulin across the blood-brain barrier and blood-spinal cord barrier in the rabbit. *J Spinal Cord Med* 18, 221-6 (1995)
128. Kaltezioti, V., G. Kouroupi, M. Oikonomaki, E. Mantouvalou, A. Stergiopoulos, A. Charonis, H. Rohrer, R. Matsas and P. K. Politis: Prox1 Regulates the Notch1-Mediated Inhibition of Neurogenesis. *PLoS Biol* 8, e1000565 (2010)
129. Gaspard, N., T. Bouschet, R. Hourez, J. Dimidschstein, G. Naeije, J. van den Amele, I. Espuny-Camacho, A. Herpoel, L. Passante, S. N. Schiffmann, A. Gaillard and P. Vanderhaeghen: An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* 455, 351-7 (2008)
130. Bové, J., M. Martinez-Vicente and M. Vila: Fighting neurodegeneration with rapamycin: mechanistic insights. *Nat Rev Neurosci* 12, 437-452 (2011)
131. Lange, C., E. Mix, J. Frahm, A. Glass, J. Müller, O. Schmitt, A.C. Schmöle, K. Klemm, S. Ortinau, R. Hübner, M. J. Frech, A. Wree and A. Rolfs: Small molecule GSK-3 inhibitors increase neurogenesis of human neural progenitor cells. *Neuroscience Letters* 488, 36-40 (2011)
132. Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodera and Y. Fukui: Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *Journal of Biological Chemistry* 269, 18961-18967 (1994)
133. Li, J., M. Siegel, M. Yuan, Z. Zeng, L. Finnucan, R. Persky, P. D. Hurn and L. D. McCullough: Estrogen enhances neurogenesis and behavioral recovery after stroke. *J Cereb Blood Flow Metab* 31, 413-25 (2011)

Abbreviations: 5-FU: 5-fluorouracil, AChE: acetylcholinesterase, BBB: blood brain barrier, CAM: cellular adhesion molecule, CASP3: caspase 3, CKK-666: 2-Fluoro-N-[2-(2-methyl-1H-indol-3-yl)ethyl]-benzamide, CKK-689: inactive version of CKK-666, CNS: central nervous system, CXCR4: chemokine receptor for SDF-1, DMSO: dimethyl sulfoxide, DNT: developmental neurotoxicity, EC50: effective concentration, ECM: extracellular matrix, ESC: embryonic stem cells, FGF2: fibroblast growth factor-2, FGF8: fibroblast growth factor-8, GABA: gamma aminobutyric acid, GTPase: guanosine triphosphate hydrolases, hESC: human embryonic stem cells, mESC: murine embryonic stem cells, HDAC: histone deacetylase, MAO-B: monoamine oxidase B, MoA: mode of action, MPP+: 1-methyl-4-phenylpyridinium, MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NPC: neural precursor cells, NO: nitric oxide, NRC: National Research Council, OECD-TG 426: Organization for Economic Co-operation and Development Test Guideline No. 426, PB: placental barrier, PCB: polychlorinated biphenyls, PNS: peripheral nervous system, PPI: prepulse inhibition, RA: retinoic acid, RKIP: Raf kinase inhibitor protein, Sema3A: semaphorin 3A, SHH: sonic hedgehog, TEP: toxicity endophenotypes, UIC1005: locostatin, UIC1017: inactive version of UIC1005

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