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EDITORIAL

Test systems of developmental toxicity: state-of-the art and future perspectives

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Reproductive toxicity: need for improved testing systems

Avoiding compounds that cause reproductive toxicity is of fundamental importance for human safety. However, reproductive toxicity testing is also one of the most challenging and expensive fields of toxicology (Wobus and Löser 2011; van Thriel 2011; Hengstler 2011). A large fraction of the animals required in drug development and in the context of REACH will be used in the area of reproductive toxicity to fulfill the respective testing requirements (Seiler et al. 2011; Krug et al. 2013; Stewart and Marchan 2012; Egbowon and Mustapha 2011; Uddin et al. 2013). Hundreds of animals are needed for testing of a single compound. Reproductive toxicity testing includes evaluation of effects on the fertilization process, spermatogenesis, oogenesis but also compromised embryo-fetal development. Currently, animal tests for developmental toxicity follow OECD guidelines 414 (2-generation study), 426 (developmental neurotoxicity) or others. These tests analyze, for example, the numbers of embryo-fetal deaths, altered total and organ weight and anatomical and behavioral abnormalities. They require exposure and analysis of animals over long periods. For example, according to OECD 426, exposure is performed during gestation and lactation and the offspring has to be analyzed for neurological, histological, neurochemical and behavioral alterations. These complex in vivo tests are too laborious and expensive to allow the required testing for thousands of chemicals (Krug et al. 2013), and might also not well reflect the human situation because of inter-species variation. Therefore, there is a general agreement that reliable, faster and more accurate in vitro tests of developmental toxicity are urgently needed (Krause et al. 2013; Leist et al. 2012; van Thriel et al. 2012).

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The novel FP7 ESNATS test systems for developmental toxicity

To improve the situation, the collaborative EU project ESNATS was conducted. ESNATS established in vitro systems that recapitulate different critical periods of human early neuronal development (Fig. 1). The test systems being named after the main involved institutions: for instance UKK, UKN1, JRC, UNIGE1 and UKN2 (Krug et al. 2013). UKK recapitulates the multi lineage differentiation of human embryonic stem cells (hESC) into ecto-, meso- and endoderm. UKN1 represents the stage of neuroectodermal induction leading to the formation of neural ectodermal progenitor cells. JRC models formation of the neural tube during early neurogenesis by the formation of neural rosettes. UNIGE1 recapitulates the transition from neural precursor cells to mature neurons. It focuses, together with UKN4, on the maturation of post-mitotic neurons and the outgrowth of neurites. As a 3D culture system, UNIGE2 closely recapitulates microarchitectural features of the central nervous system.

UKN2 uses neural crest cells generated from human embryonic stem cells (hESC) and examines their functional properties (Zimmer et al. 2012).

Standard operation procedures (SOPs) of all test systems are available (Krug et al. 2013). To consider metabolism, the in vitro systems have been combined with cultivated human hepatocytes. It has been demonstrated that inclusion of hepatocytes may enhance toxicity by more than 100-fold or strongly reduce toxic effects in the target cells depending on the type of test compound. To identify in vivo relevant test compound concentrations, techniques of modeling have been improved by integrating metabolic, PBPK and spatio-temporal tissue models (Hoehme et al. 2010; Zeigerer et al. 2012). All test systems have been established in close cooperation with pharmaceutical companies and with regulatory authorities. The starting cells of the novel FP7 ESNATS test systems are either human embryonic stem cells (hESC) or neuronal precursor cells abbreviated above hESC. As far as hESC are involved, pilot experiments have been successfully performed to establish test systems also on the basis of induced pluripotency stem cells (iPSC).

Specific signatures identify DNT compounds

The novel FP7 ESNATS test systems were exposed to two classes of compounds known to cause developmental neurotoxicity (DNT). Valproic acid (VPA) and related compounds cause neural tube defects while the human neurotoxicity and DNT of methylmercury (MeHg) has been well documented due to catastrophic endemies caused by contaminated food. Analyzing the gene expression alterations induced by both test compounds allowed a clear differentiation from negative control compounds (here: Mannitol and DMSO, respectively) and from each other (Fig. 2).

This success encouraged the ESNATS consortium to perform a blinded classification study using six compounds acting either by ‘valproic acid like mechanisms’ (histone deacetylase inhibitors) or by mechanisms similar to methylmercury. Classifiers could be established that clearly differentiate the DNT compounds from their solvent controls. This is remarkable, considering that simpler cell systems, such as fibroblasts or even neuronal cell lines do not allow a sufficient distinction. Genome-wide analyses also made clear that our current categories of DNT, e.g., histone deacetylase (HDAC) inhibitors, mercurials, kinase inhibitors, etc., may not be sufficient to correctly describe the influence of chemicals on the developing central nervous system. Most probably, extended analyses will lead to novel categories and classification systems. The ESNATS proof-of-concept study clearly demonstrates the importance of cell systems that recapitulate critical processes of human development. Exposure to test compounds in vitro must be performed exactly during time windows when such developmental steps take place. In this case, stress response pathways and adverse outcome pathways (AOPs) have been derived from the deregulated genes. For both compound classes AOPs associated with disturbed neuronal development are now available.

Future directions

Deeper understanding of the test systems

One of the reasons for the success of the ESNATS test systems is that a relatively high effort has been invested to guarantee that the in vitro systems recapitulate relevant processes of human central nervous system development. Should the consortium have chosen an approach with easier already available cell systems and a screening of hundreds of compounds, this approach would most probably have failed. Nevertheless, an even deeper understanding of the established test systems is urgently needed. For example, neuronal differentiation in the ESNATS test systems is characterized by tightly coordinated waves of gene expression (Schulz et al. 2009; Zimmer et al. 2011; Gaspar et al. 2012). This feature of the differentiating stem cells recapitulates expression waves of the developing central nervous systems in vivo. Complex modeling and systems biology approaches will be needed to understand how such ‘waves of development’ are coordinated and how they can be perturbed by toxic compounds. It is also critical to understand how these perturbations are linked to adverse effects in vivo. This leads to a critical aspect of EU-funding policy. In previous projects, funding has been limited

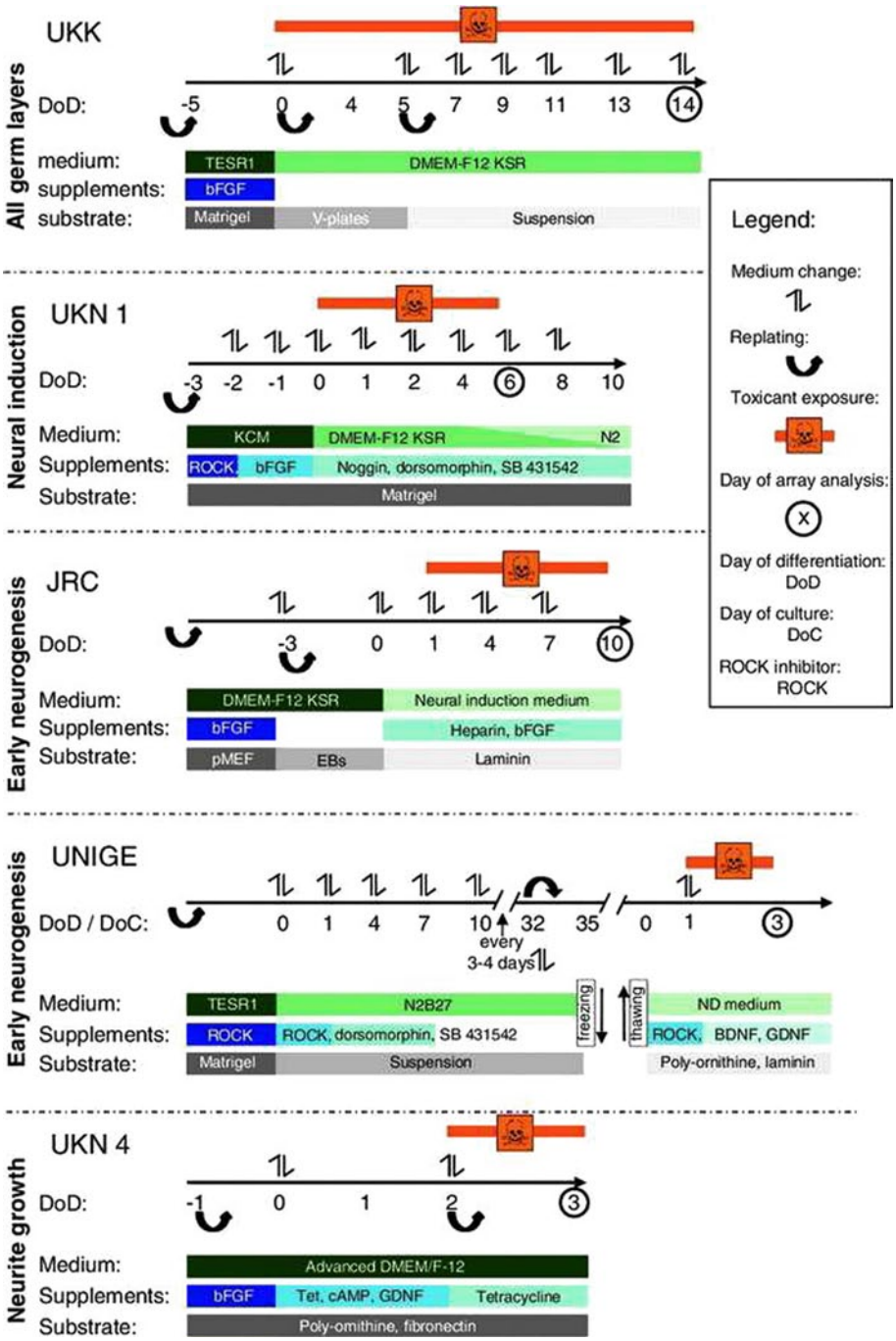

Fig. 1 Overview over the novel FP 7 ESNATS test systems for developmental neurotoxicity. The five test systems cover different periods and processes relevant to early embryonic/neuronal development, as indicated to the *left*. The time *arrows* indicate when cells were replated, medium was exchanged, toxicants were added and analysis was performed (from: Krug et al. 2013)

to human in vitro cell systems. However, to achieve a better understanding of the in vivo relevance of 'developmental waves' in vitro, it should be possible to compare them with the in vivo situation. In vivo data are also required to understand how disturbance of 'developmental waves' are linked to adverse effects. Such an understanding could be achieved by comparing developing mouse in vitro systems with mouse in vivo data. This would help to better interpret data of the corresponding human in vitro systems, such as those established by ESNATS. Therefore, future research programs aimed at improving human safety assessment and replacing animal experiments would benefit from inclusion of well-justified supplementary research in rodents and rodent cells, besides human cell systems, in order to guarantee that the in vitro systems indeed recapitulate the most critical steps in vivo.

Reducing complexity and modeling

A central result of ESNATS is that DNT compounds cause specific patterns of gene expression alterations in the novel

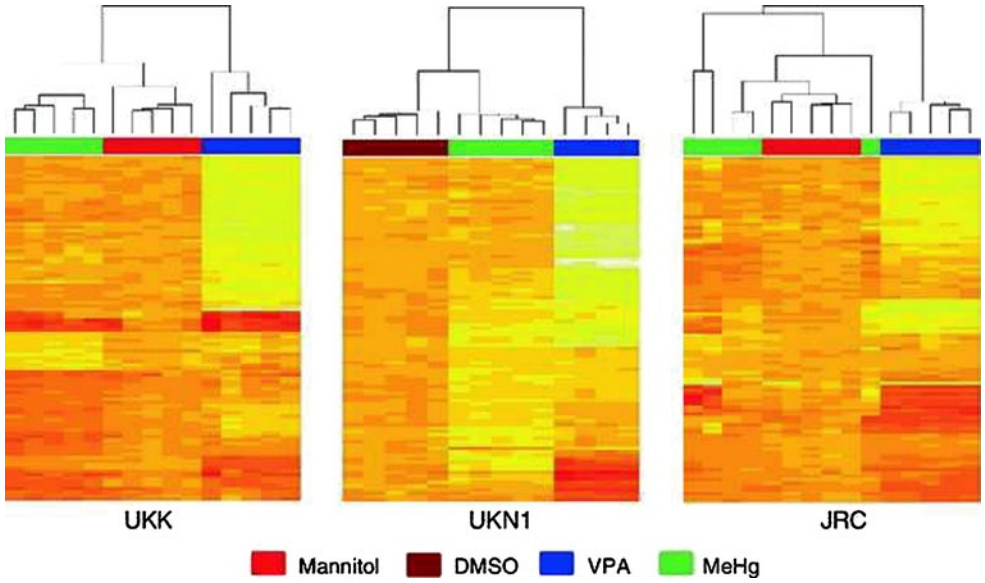

Fig. 2 Heatmap analysis of gene expression alterations in the FP7 ESNATS test systems. VPA and MeHg are representatives of two classes of compounds known to cause developmental neurotoxicity in humans. Gene expression alterations induced by both compounds are clearly distinct and also differ from those of corresponding negative control compounds (from: Krug et al. 2013)

FP7 ESNATS test systems of developmental toxicity (Krug et al. 2013). To interpret these patterns, software for identification of over-represented biological motifs is usually applied. One result of the ESNATS project is that identification of the transcription factors responsible for the compound-induced gene expression alterations is an efficient strategy to reduce complexity. While some transcription factors indicate a general stress response, others seem to be linked to more specific toxic processes. In future, a close cooperation between experimentalists, biostatisticians and modelers is required to decipher the complex expression patterns and understand their relationship to adverse effects in vivo.

Compound screening and validation studies

A final goal of in vitro test systems development is the determination of sensitivity and specificity after analysis of large numbers of positive and negative compounds. However, on the way to this ultimate goal, pitfalls should be avoided. One danger is to initiate large screening programs too early. First, we have to answer the question whether the battery of available in vitro systems covers a sufficient number of mechanisms and processes relevant to in vivo toxicity. In the FP7 ESNATS test systems, this has been shown for only two classes of DNT compounds, namely the VPA and methylmercury type of compounds. It is difficult to predict whether these in vitro systems cover already all relevant mechanisms of developmental neurotoxicity. Considering the high complexity of the CNS, this seems rather unlikely. Therefore, a stepwise strategy of optimization seems to be most promising. First, further compounds with known developmental neurotoxicity but acting by other mechanisms than VPA and methylmercury should be tested. It will be particularly relevant to further progress, if compounds can be identified that trigger new patterns of toxicity in the so far established FP7 ESNATS in vitro systems. In this case, the critical in vivo mechanisms leading to toxicity must be identified. In the future, also the question has to be addressed whether there are mechanisms of toxicity that are not sufficiently represented in the available in vitro systems. Possibly, additional cell systems or improved in vitro techniques have to be established. Only when this process will have been convincingly accomplished, large studies for determination of sensitivity and specificity and formal validation studies will make sense. Successful establishment of in vitro systems can only be an iterative process with many cycles of improvement and comparisons to processes in more complex settings (gold standards). The classical gold standard of the past has been rodent in vivo studies. It remains to be seen whether human cell-based 3D tissues may not be more suitable and reliable as far as human prediction is concerned (Table 1).

Conclusions

Human stem cell-based in vitro test systems have been established in ESNATS that recapitulate relevant processes of the developing human central nervous system.

Table 1 Achievements of ESNATS and future directions

Achievements of the ESNATS consortium	Future perspectives
Novel in vitro systems have been established that recapitulate critical processes of human central nervous system development; standardization of test systems and sufficient reproducibility have been accomplished	Analyses of broader sets of test compounds must show if all in vivo relevant processes of DNT are represented; eventually optimizations will be required; hESC-based systems may be replaced by iPSC technologies
Procedures of handling of genome-wide complex data have been optimized and standardized: normalization based on optimized frozen RMA, cluster identification, recognition of biological motifs, stability analyses and identification of over-represented transcription factors	Future studies will have to identify the most efficient and accurate techniques of complexity reduction; e.g., are transcription factor-based classification systems superior over gene-based classifiers?
Classifiers for identification of DNT compounds are available; a blinded classification study correctly differentiated DNT compounds from negative controls	Current textbooks do not adequately categorize DNT compounds. Novel more accurate classification systems of DNT and DT have to be developed
The human hepatic metabolism has been included by cultures of primary human hepatocytes and culture medium transfer. Improved techniques of metabolic modeling are available.	Besides the available 'medium transfer techniques' more direct technologies of metabolite transfer to the target cells are needed, eventually based on the 'body-on-a-chip' principle
PBPK-based techniques for analyses of in vivo relevant concentration are available; in vitro–in vivo extrapolation to the prenatal situation is possible. PBPK modeling has been integrated into spatio-temporal models.	The precision of in vitro-in vivo extrapolation of test compound concentrations must be improved and confirmed, including in vivo analyses of test compound and metabolite concentrations as well as the possibility to predict in vivo concentrations by modeling
The basic principles of concentration and time-resolved compound effects are understood; e.g., unspecific toxicity associated signatures (such as downregulation of metabolic functions) can be differentiated from specific events of dysregulated neuronal development. 'Waves of development' in vitro show a high degree of similarity to the in vivo situation.	Control mechanisms of 'waves of development' and their susceptibility to chemicals still have to be understood and modeled; a causal understanding of disturbed expression waves and adverse effects in vivo still has to be established.

A proof-of-concept study demonstrated that compounds causing developmental neurotoxicity can be identified in these systems. Further, projects should be initiated to study a broader range of chemicals and to optimize the test systems. It has become clear that stem cell-based in vitro systems will become an accurate, fast and cost-effective tool for identification of toxic compounds in the broad field of developmental toxicity. This will be a major contribution to human safety.

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