Briefing paper

The novel FP7 ESNATS test systems of developmental toxicity: state-of-the art and future perspectives

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. 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). 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-foetal 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-foetal deaths, altered weight, 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.

The novel FP7 ESNATS test systems for developmental toxicity

To improve the situation, the collaborative EU project ESNATS was performed. 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 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 hESC and examines their functional properties.

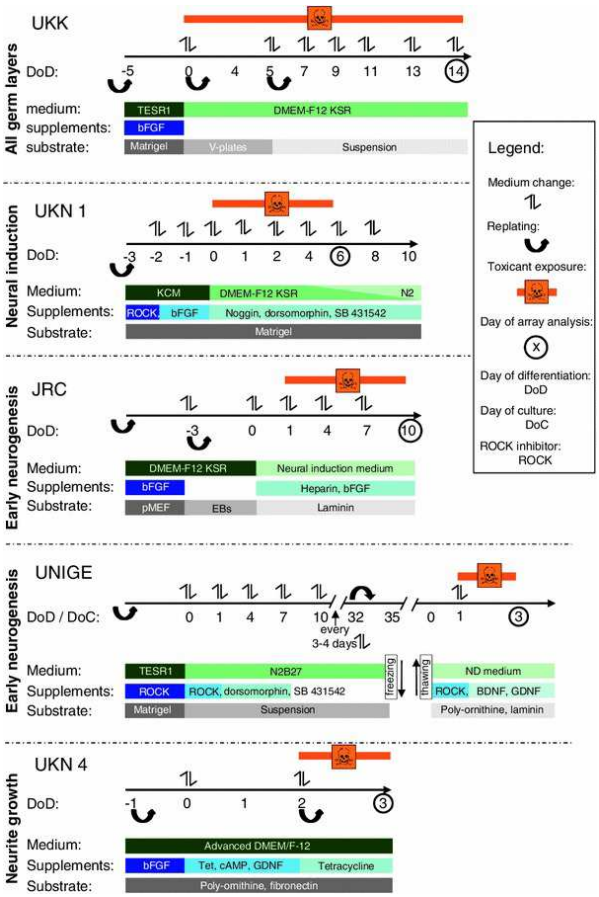

Fig. 1: Overview over the novel FP 7 ESNATS test systems for developmental neurotoxicity. The five test systems cover different periods and processes relevant to early embryonic/neuronal development, as indicated to the *left*. The *time arrows* indicate when cells were re-plated, medium was exchanged,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

Standard operation procedures 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 spatial-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 neuronal precursor cells or embryonic stem cells (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 and related compounds cause neural tube defects while the human neurotoxicity of methylmercury 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 from each other (Fig. 2).

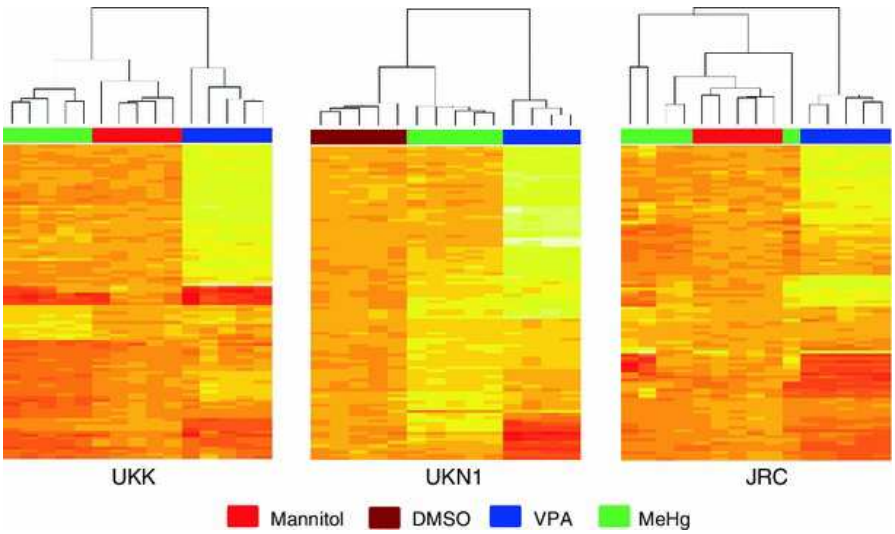

Fig. 2: Heatmap analysis of gene expression alterations in the FP7 ESNATS test systems. Valproic acid (VPA) and methylmercury (MeHg) are representatives of two classes of compounds known to cause developmental neurotoxicity in humans. Gene expression alterations induced by both compounds are clearly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

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. 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 (AOP) 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, 2009; Zimmer, 2011; Gaspar 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 disturbed by toxic compounds. It is also critical to understand how these disturbances are linked to adverse effects *in vivo*. This leads to a critical aspect of EU funding policy. In previous projects, funding has been limited 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 to 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 to 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 i*n vivo*.

Reducing complexity and modeling. A central result of ESNATS is that DNT compounds cause specific patterns of gene expression alterations in the novel FP7 ESNATS test systems of developmental toxicity (Krug et al., 2013). To interpret these patterns, software for identification of overrepresented 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 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 for *in vivo* toxicity. In the FP7 ESNATS test systems this has been shown for only two classes of DNT compounds, namely the valproic acid 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 valproic acid and methylmercury should be tested. It will be particularly relevant for 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 mechanism 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 have 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.

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. 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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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 overrepresented 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 text books 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 spatial-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 has to be understood an modeled; a causal understanding of disturbed expression waves and adverse effects *in vivo* still has to be established.

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