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Review article

Relevance of *in vitro* neurotoxicity testing for regulatory requirements: Challenges to be considered

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ABSTRACT

The current testing requirements for both adult and developmental neurotoxicity evaluation are based on *in vivo* animal models and the neurotoxic potency of compounds is mainly determined by neurobehavioural and neuropathological effects. *In vitro* studies are considered complementary to animal tests because they provide an understanding of the molecular/cellular mechanisms involved in neurotoxicity. However, the selection of relevant *in vitro* neuronal/glial specific endpoints applied to various neuronal cellular models should be done in a careful way to build reliable and feasible testing strategies since usually these endpoints have to be tested in various complementary *in vitro* systems. The requirements for applying a more complex test strategy where toxicokinetic aspects are included together with different tools to compensate for the lack of *in vitro* metabolic competence are discussed. Taking into consideration the recent European Commission chemical legislation concerning registration, evaluation and authorisation of chemicals (REACH) it has become a priority to develop new intelligent testing strategies integrating computational models and *in vitro* assays based on cell culture models and endpoints that are amenable for adaptation to high throughput screening to be able to test a large number of chemicals.

Contents

1. Introduction

Neurotoxicity detection induced by chemicals represents a major challenge due to the physiological and morphological complexity of the central (CNS) and peripheral nervous system (PNS). Currently regulatory authorities such as the Organisation for Economic Co-operation and Development (OECD) and the U.S. Environmental Protection Agency (U.S. EPA) use solely animal *in vivo* methods for both adult and developmental neurotoxicity testing [34,49]. Neurotoxicity is evaluated during acute systemic toxicity, repeated-dose toxicity, subchronic, chronic and reproductive/developmental toxicity.

The developing human brain can be more susceptible to injury caused by toxic agents than the brain of an adult. Probably all potential neurotoxic compounds would also cause damage to the developing brain and at much lower doses [21,48]. Indeed, neurodevelopmental disorders in children such as attention deficit disorder, mental retardation or autism have been associated with the exposure to chemicals in the environment during early fetal development [18,31,42].

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Currently *in vitro* tests are generally used to study the mechanisms of toxicity rather than to detect neurotoxicity for prediction of hazards to human health and so far they play only a complementary role to *in vivo* testing. *In vivo* test methods that are used for neurotoxicity evaluation of the developing nervous system (OECD TG 426) [35] as well as the matured nervous system (TG 424) [36] are mainly based on neurobehavioral evaluation of cognitive, sensory and motor functions accompanied by neuropathological studies. However, due to the actual need to test large sets of compounds for specific regulatory requirements in Europe and the U.S. (e.g. the REACH policy and the High Production Volume Programme (HPVP) there is a high pressure to develop alternative test strategies which are more rapid, economically feasible and have an acceptable predictive capacity [2].

A combination of *in vitro* assays could be used to identify alerts specific for neurotoxic compounds, improving the assessment and predictability for human neurotoxicity. However, before *in vitro* assays could be incorporated into an intelligent testing strategy for human neurotoxicity evaluation, they would have to undergo a validation process to prove that they are neuronal and glial specific, robust and preferably amenable to high throughput screening.

Implementation of *in vitro* neurotoxicity tests, as stand-alone or as part of an integrated test strategy where a battery of *in vitro* mechanistic assays is incorporated, would accelerate the process of testing by delivering mechanistic data on chemical-induced toxicity. However, until now no *in vitro* approaches for evaluating the neurotoxic hazard of compounds have been formally validated. To speed up the validation process and the incorporation of existing *in vitro* models and endpoints into neurotoxicity testing strategies, the existing bottle-necks of the current approaches have first to be identified and then possible solutions suggested.

In this review the authors from European Centre for the Validation of Alternative Methods (ECVAM) discuss some of the challenges associated with *in vitro* neurotoxicity testing including developmental neurotoxicity in order to invite the scientific community to use the current knowledge and technologies to progress towards the establishment of predictive strategies for human neurotoxicity assessment which are acceptable for governmental agencies.

2. Critical mechanisms of neurotoxicity should be covered by neuronal specific endpoints using complementary *in vitro* models

So far, the test systems developed for *in vitro* neurotoxicity assessment for regulatory purposes have often been based on general cytotoxicity assays and do not sufficiently represent the endpoints specific for mechanisms of neuronal and glial toxicity. Cytotoxicity assays can be applied as an initial "screening" to establish the concentration-dependent curve (IC 20, 50 and 80) and to define the non-cytotoxic concentrations that would later be tested using neuronal and glial specific endpoints. Such a testing strategy would establish whether non-cytotoxic concentrations could already induce neurotoxicity that has to be determined by neuronal or glial specific endpoints [2]. Therefore, *in vitro* neurotoxicity testing should firstly determine cell viability/death (cytotoxicity assays) followed by critical but general cell function tests such as energy metabolism, glucose uptake, oxidative and nitrosative stress, calcium homeostasis etc. and finally should be focused on neuronal specific cell function endpoints for example electrical activity, neurotransmission, axonal transport, receptor and channel activation, enzyme activity, synaptogenesis/myelination, excitotoxicity, and neuronal–glial interactions. So far *in vitro* neurotoxicity testing is mainly used for mechanistic studies where molecular/cellular pathways of toxicity are determined and used as the readout of chemically induced neuronal and glial damage. However, a large research project ACuteTox [11] sponsored by the European Commission which focused on the integrated approach for acute toxicity testing, including acute neurotoxicity, proved that the selected neuronal endpoints should reflect general neuronal functional perturbations induced by chemicals resulting from underlying molecular changes. Moreover, it is possible that some observed molecular modifications induced by compounds could be compensated for with time or overcome by defence mechanisms and perhaps it would be more reliable to study the final functional outcome of such changes. It is proposed that the general (but still neuronal specific) functional endpoints associated with known biological mechanisms of toxicity, if possible, should refer not to a particular neuronal cell type of a specific structure in the brain but rather should be expressed by all neuronal cell types (e.g. measurements of electrical activity). Perhaps, by focussing on the general neuronal functional endpoints it would be possible to replace the long list of endpoints that are based on detailed molecular mechanisms to a few assays but still covering most of the aspects critical for neuronal function. A similar approach could be applied to developmental neurotoxicity testing (DNT) and selected endpoints should refer to the key processes which are important for neurodevelopment instead of applying an extensive list of molecular and mechanistic endpoints. However, a distinction on whether the toxic agent affects neurons, astrocytes or oligodendrocytes (the three main cell lineages of the CNS) is necessary. This is especially important in DNT evaluation, because critical cellular events in developing nervous system (including neural cell type commitment) are precisely temporarily and spatially coordinated [19,47] and specific windows of vulnerability for differentiating neurons, astrocytes and oligodendrocytes may not overlap [43,1]. The critical developmental processes recommended as endpoints for DNT was published in a report from an international workshop, co-organised by the European Chemical Industry Council, the European Centre for the Validation of Alternative Methods and the Johns Hopkins Center for Alternatives to Animal Testing in the U.S. [17]. The list includes the early developmental processes such as proliferation, migration, apoptosis and neural cell type commitment and more advanced stages such as differentiation of precursor cells, neurite outgrowth, glia activation, myelinisation and electrophysiological activity [17]. Human embryonic stem cells and neural cell lines have recently become available, in which several of these processes can be studied. Normal Human Progenitor cells (NHNP) grown as neurospheres (Cambrex Bioscience) have been used to study cell specific protein expression and processes of migration after chemical exposure [22,28], whilst the immortalised Human Neural Progenitor Cell Line (ReNcell CX) has been shown to be a good model for studying toxic effects on cell proliferation and viability [5,9]. Human embryonic stem cells were used to investigate the effects of toxicants on neuronal differentiation whereas the Human Umbilical Cord Blood Derived Neural Stem Cell line (HUCB-NSC) [8] was used to study developmental processes such as proliferation, apoptosis, neural differentiation and electrophysiological activity [7,27]. It is important to emphasis that these are human models, therefore they avoid the need for interspecies extrapolation of results, which is not always straightforward.

3. The anatomical and physiological complexity of PNS and CNS should be represented by relevant *in vitro* systems

CNS and PNS are the systems with various cell types organised into a functional network that is required to maintain an integrated function of the nervous system.

Due to its complexity none of the existing *in vitro* models entirely reflect the *in vivo* situation as once isolated most of the neuronal culture systems represent cells that are no longer part of any integrated neural network. The isolated cells may develop an altered appearance, function, different level of cell–cell interaction and usually have dramatically decreased metabolism that could result in an altered response to test chemicals when compared with the *in vivo* situation. Additionally, the *in vitro* test systems currently available cannot be used to assess the neurobiological *in vivo* functions such as i.e. cognition, motor coordination, sensory processing and integration