Neurotoxicity Testing: A Discussion of *in Vitro* Alternatives

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A large number of chemicals may exert adverse effects on the central and/or peripheral nervous system. A commonly recommended strategy for neurotoxicity testing is that of a tiered approach aimed at identifying and characterizing the neurotoxicity of a compound. Guidelines exist in the United States and other countries that define the tests to be utilized in tier 1 testing. To address problems related to the increasing cost and time required for toxicity testing, the increasing number of chemicals being developed, and the concern of animal welfare activists, attention is currently being devoted to *in vitro* alternatives. This paper addresses the use of *in vitro* systems in neurotoxicology, and their potential role in a general strategy for neurotoxicity testing. The advantages and disadvantages of *in vitro* approaches for mechanistic studies and for screening of neurotoxicants are discussed. Suggestions for further validation studies are proposed. — *Environ Health Perspect* 106(Suppl 2):505–510 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/505-510costa/abstract.html*

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Introduction

The nervous system is one of the most complex organ systems in terms of both structure and function. Nerve cells are unique in that they are not capable of regeneration after lethal damage, thus rendering the nervous system particularly vulnerable to toxic insult (*1,2*). Neurotoxicity is defined as "any adverse effect on the chemistry, structure and function of the nervous system during development or at maturity, induced by chemical or physical influences" (*3*). The key issue is the interpretation of the word adverse, as there is not always agreement among scientists on what constitutes an adverse change. Issues that have been considered include the nature of the change (morphologic, neurochemical, neurologic, or behavioral), the degree of change, and whether the effect is transitory or persistent. A proposed definition of an adverse effect is "any treatment-related change which interferes with normal function and compromises adaptation to the environment" (*4*). Most morphologic changes such as neuronopathy, axonopathy, or myelinopathy would be considered adverse, even if the changes were mild or transitory. On the other hand, a transitory hypertrophy of astrocytes could be viewed as an adaptive, physiologic response (*4*). However, one could argue that such an adaptive response may reflect prior damage to neuronal structures, and thus be a relevant biomarker of an adverse effect. Furthermore, a still-debated issue is whether neurochemical effects in the absence of structural damage should be considered adverse. Certainly an acute intoxication with an organophosphorus insecticide produces acute neurotoxic effects that tend to resolve with time. Additionally, repeated exposure to these compounds changes the homeostasis of the cholinergic system, altering the response to cholinergic drugs and possibly interfering with cognitive processes (*5*). Similarly, acute response to solvents may cause only transient central nervous system effects, but these should be considered neurotoxic as they can lead to impaired function.

Another concept in neurotoxicity is the difference between direct and indirect effects on the nervous system. Damage to hepatic, renal, or pancreatic structures can result in secondary effects on the function and structure of the nervous system, such as encephalopathy or polyneuropathy. Secondary effects would not cause a substance to be considered neurotoxic; however, at very high doses, neurotoxicity would be evident. The identification of neurotoxic effects seen after administration of doses that exceed the maximum tolerated dose, or at which the normal metabolic processes of the body are severely compromised, is therefore a recognized problem (*4*). Thus, a substance should be defined as neurotoxic when it or its metabolites produce adverse effects as a result of direct interactions with the nervous system. This paper briefly reviews some current approaches and methods of *in vivo* neurotoxicity testing and their advantages and limitations. It then analyzes the current efforts aimed at implementing and validating *in vitro* methodologies for such testing, and discusses the steps necessary for an integration of the different approaches.

Detecting Neurotoxicity in Standard Toxicity Testing

Standard toxicity studies, required by national and international regulatory agencies, involve exposure to a chemical on an acute, subacute, subchronic, and chronic basis, in addition to reproductive studies and other studies (e.g., those for genotoxicity). These studies are relevant in the assessment of potential neurotoxicity, as they are conducted at different dose levels, with different routes and durations of administration, in different animal species. Data on metabolism and toxicokinetics are also developed in support of such studies. These studies include clinical observations and morphologic examinations, which can reveal effects on the nervous system. These may include changes in motor functions or in autonomic functions, or damage to relevant brain structures. It is important that such studies are conducted carefully and the observations documented precisely. Clinical signs should be recorded as they are observed, and should include time of onset, degree, and duration of effects. Parameters should include changes in skin, fur, secretion, activity, gate, response to stimuli, etc. Brain weight should be recorded at autopsy. Histologic examination should include the brain, the spinal cord, and one peripheral nerve. Examples

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Abbreviations used: OECD, Organisation for Economic Co-operation and Development; OPIDN, organophosphate-induced delayed neuropathy; U.S. EPA, U.S. Environmental Protection Agency.

of chemicals whose neurotoxicity has been identified in standard subchronic studies include sulfuryl fluoride (*6*), tris(2-chloroethyl)phosphate (*7*), and benzaldehyde (*8*). If the clinical observations and morphologic examination do not indicate any evidence of nervous system effects, and when the chemical structure of the substance and/or its metabolites does not suggest concern for potential neurotoxicity, the substance can be considered as not neurotoxic. In this case, no further testing would be warranted until any new cause for concern for the nervous system may arise. If standard toxicity studies of a new chemical provide evidence of direct, adverse effects on the nervous system, its development for use may be abandoned without further testing (*2*). On the other hand, neurotoxicity testing may be conducted for more commonly existing substances with commercial value or wide exposure.

Testing for Neurotoxicity: A Tiered Approach

Because of the variety of toxic effects and of cellular biochemical targets, a test strategy for the evaluation of the neurotoxic potential of chemicals should not be totally rigid, but should be determined case by case, with an emphasis on the structure of the chemical and the indications of the standard toxicity tests. Though structure-activity relationship studies are undoubtedly important, caution should also be exerted, as empirical findings can be unexpected and unpredictable at times. For example, among organic tin compounds only two—trimethyltin and triethyltin—are neurotoxic, though their effects are notably different, whereas other trisubstituted compounds are not.

A commonly recommended strategy for neurotoxicity testing is that of a tiered approach consisting of three tiers aimed at identifying (tier 1), and characterizing (tiers 2 and 3) the neurotoxicity of a chemical (*2*). Initial neurotoxicity studies should thoroughly evaluate the nervous system with a number of broad but system-specific tests, to provide adequate data for risk assessment. Because of the multiplicity of possible effects on the nervous system, there is no single test method that can ensure the detection and identification of every possible change. The tests that are used are those included in the guidelines of the U.S. Environmental Protection Agency (U.S. EPA) (*9*) and the Organisation for Economic Co-operation and Development (*10*). These include acute and subchronic administrations of the tested chemical and a number of behavioral observations, specific measurements, and neuropathologic examinations (Table 1). The functional observation battery is a standardized screening battery for the assessment of many aspects of behavior and neurologic functions in rodents, and has been validated with several known neurotoxic chemicals. Special tests are conducted to identify specific deficits in motor and sensory functions; these may include automated measurements of motor activity, rotating rods, landing footspread, measurements of fore- and hindlimb strength, measurements of auditory or thermal thresholds, and measurements of startle reflex.

Histologic examination of central and peripheral nervous systems would include as a minimum the following tissues: brain (several sections including those structures involved in specific types of behaviors), spinal cord (including dorsal root ganglion and dorsal/ventral roots), and peripheral nerves (sciatic and tibial nerves). Measurement of glial fibrillary acidic protein or similar approaches (e.g., involving measurements of neuronal-specific proteins or microglial reactivity), would also provide useful information on potential neurotoxicity, particularly if the experimental design provides for multiple measurements (*29*).

If there is no evidence of a nervous system effect in tier 1 studies, then the substance can be considered as not neurotoxic. When, however, persistent effects on the nervous system are detected, the chemical can be considered a probable human neurotoxicant. In such cases, additional tests may be warranted in tier 2 and 3 studies to characterize the neurotoxic effects. The data from tier 1 studies should provide the basis for generation of specific hypotheses for subsequent studies and guide in the selection of the appropriate methods. The decision to carry out additional studies should be made on a case-by-case approach, and may depend upon factors such as the intended use of the chemical, the potential of human exposure, and its potential to accumulate in biological systems. Such studies may include specialized behavioral tests, electrophysiologic and neurochemical measurements, and additional morphologic studies. Examples of such studies include tests for measuring learning and memory (active or passive avoidance, spatial discrimination tasks); operant conditioning with various schedules of nerve conduction velocity; electromyography, neurochemical tests (markers of neuronal or glial cells, biochemical parameters of cell functions or integrity, biochemical parameters related to neurotransmission and signal transduction); measurement of 2-deoxyglucose accumulation; and analysis of nerve tissue after perfusion fixation (which may include electron microscopy examinations and use of immunohistochemical and morphometric methods). These tests should allow a thorough characterization of the neurotoxicity of a substance and provide information on its mechanism of action.

Table 1. Summary of the U.S. EPA neurotoxicity testing guidelines.

Animals
Adult rats, male and female
Group size
10/sex/group
Control group
Vehicle control
Dose level
Acute exposure (3 doses)
Subchronic exposure (3 doses)
Route of exposure
Consider human exposure kinetics
Observations
Convulsions, tremors, hyperactivity, aggression, stereotypes, autonomic signs, abnormal posture or gait, activity level, abnormal respiration, vocalization
Measurements
Body weight
Sensory functions
Grip strength
Motor activity
Landing foot splay
Neuropathology
In situ fixation
Paraffin/plastic embedding
H&E stain
Additional stains if necessary
Tissues/sections
All major regions of nervous system (central and peripheral)

Special Issues in Neurotoxicity Testing

Neurotoxicity Induced by Organophosphorus Compounds

A number of organophosphorus compounds that have been used mostly as insecticides have the ability to cause a particular neurotoxicity known as organophosphate-induced delayed neuropathy (OPIDN). OPIDN is characterized as a distal–central peripheral neuropathy and becomes apparent with a delay of 2 to 3 weeks after acute exposure. Regulatory guidelines require that specialized neurotoxicity testing be conducted with all compounds of this class (*9,10*). The compound is administered acutely and

subchronically to adult hens and the eventual insurgence of ataxia and other clinical signs are monitored and scored. Neuropathologic examinations of brain, spinal cord, and peripheral nerves are then carried out. The U.S. EPA also requires biochemical measurement of the activity of an enzyme, neuropathy target esterase, that is considered the target for initiation of the neuropathy. For several years, testing for OPIDN has been the only type of neurotoxicity testing required by regulatory agencies, and the process has been, for the most part, successful in detecting compounds able to cause delayed neurotoxicity and prevent their commercialization. Recently, however, neurotoxicity occurred in an individual who had ingested an extremely high dose of an organophosphate (far exceeding the dose used in animal studies) and was able to survive the acute cholinergic poisoning.

Developmental Neurotoxicity

The nervous system undergoes gradual development that continues well after birth in both animals and humans. On one hand, the developing nervous system can more readily adapt to, or compensate for, functional losses as a result of toxic insult; however, these may result in a delayed functional development. On the other hand, damage to the nervous system during key periods of brain development may result in long-term, irreversible damage. The absence of a fully developed blood-brain barrier may result in the accumulation in the brain of certain chemicals in greater quantities than in adults. Special guidelines thus exist to determine developmental neurotoxicity. In principle, carefully conducted reproductive studies should be able to provide initial indications of potential developmental neurotoxicity. However, it has been shown that reliance on, for example, the Chernoff/Kavlock teratology assay as a primary developmental neurotoxicity screen could lead to a number of false negatives (*30*). Current developmental neurotoxicity screening (*9*) includes a number of observations and measurements aimed at determining the development of proper motor sensory functions, learning, and memory, together with landmarks of physical development.

In Vitro Neurotoxicity Testing

The need to develop acceptable alternatives to conventional animal toxicity testing is increasingly recognized by toxicologists, to address problems related to the escalating costs and time required for toxicity assessment, the increasing number of chemicals being developed and commercialized, and the concern of animal-welfare activists (*11,12*). In the past several years, a number of publications have addressed issues related to the use of *in vitro* systems for neurotoxicity testing; they are reviewed here only briefly (*11,13–18*).

In vitro testing procedures have several practical advantages; yet, the limitations of such approaches should also be outlined, particularly in the field of neurotoxicology, because of the complexity of the nervous system. To date, *in vitro* systems have been used extensively to study the mechanism of action of neurotoxic agents. However, their use in hazard identification in human health risk assessment has not been explored to any great extent (*19*). Data generated from *in vitro* procedures are based on simplified approaches that require less time and cost to yield information (two clear advantages), but that do not take into account the distribution of the toxicant in the body, the route of administration, and the metabolism of the substance (*19*). In addition, it is difficult to extrapolate *in vitro* toxicity data to animal or human neurotoxicity end points such as sensory disorders or cognitive impairment. Several *in vitro* systems are currently being evaluated for their ability to predict the neurotoxicity of various agents seen in intact animals, and an important part of this process should be the detection of false negatives. Until this process proves satisfactory, a cautious view should be that outlined recently by the U.S. EPA in their proposed guidelines for neurotoxicity risk assessment: "Demonstrated neurotoxicity *in vitro* in the absence of *in vivo* data is suggestive but inadequate evidence of a neurotoxic effect. On the other hand, *in vivo* data supported by *in vitro* data enhance the reliability of the *in vivo* results" (*19*).

What are the advantages of *in vitro* testing systems for neurotoxicity, and what is their best utilization in neurotoxicology? The savings in terms of time, costs, and animal use have already been mentioned. Other advantages of *in vitro* toxicity testing are summarized as follows:

- Uniform chemical and physical environment
- Toxic exposure continuous or intermittent
- Exposure parameters strictly controlled
- Small amounts of chemical needed
- Systemic (e.g., hepatic) effects bypassed
- Range of donor species available
- Human materials available (*11*)

Limitations of *in vitro* toxicity testing are *a*) lack of integrated functions and blood–brain barrier function; *b*) target concentration not known; *c*) compensatory mechanisms cannot be determined, and *d*) single tests cannot cover all targets and mechanisms (*3*).

Most *in vitro* systems for neurotoxicity make use of mammalian cells. In decreasing order of complexity, these models include organotypic explants, brain slices, reaggregate cultures, primary cell preparations, and established cell lines. It should be noted that with the exception of cell lines all other models involve the use of cells or tissues directly derived from animals. Thus, while the number of animals may be reduced (as *in vitro* systems allow the testing of multiple concentrations of chemicals and other experimental manipulations in tissue derived from a single animal), other problems inherent to the use of primary cell or tissue cultures still necessitate the use of a substantial number of animals. From the point of view of reducing the number of animals, established cell lines are certainly the system of choice. A benefit of using organotypic explants, brain slices, or reaggregate cultures is that the cytoarchitecture of the nervous system, the neural circuitry of a specific brain area, or other biochemical processes (e.g., myelination), are preserved. Systems using primary cultures, on the other hand, do not offer the retention of neuronal circuitry but allow the study of the effects of toxicants on separate cell types (e.g., neurons, astrocytes, oligodendrocytes). The latter results can also be achieved by the use of established cell lines. These have been derived from tumors of mouse, rat, and human, and are, for the most part, available commercially. Cell lines are usually the simplest to culture and many (e.g., several neuroblastoma or glioma cell lines or PC12 cells) have been extensively used in neurobiologic studies, thus providing a large amount of information on their physiology and biochemical composition. Furthermore, reproducibility of results is often better in cell lines than in primary cultures. On the other hand, cell lines are transformed (tumor-derived) cells; thus, effects in these cells may not always mimic those occurring in untransformed cells.

In Vitro Systems for Mechanistic Studies

In vitro systems are amenable and very useful for mechanistic studies at the cellular and molecular level. As such, they have

been extensively used in neurobiology and, to a minor extent, in neurotoxicology. Because of the variety of nerve cells and the complexity of the nervous system, no single *in vitro* preparation can be relied on to detect all possible end points (*11,13*). Depending on the status of knowledge on the neurotoxicity of a certain compound, and of specific questions that are being asked, different cellular systems or preparations can be used, and a tiered approach can be applied in this context as well. For example, neuroblastoma or glioma cell lines can be used to investigate the interaction of neurotoxicants with ion channels or receptor and signal transduction systems, as well as with basic metabolic functions. The hippocampal slice preparation can be used to determine whether a chemical may affect certain excitatory or inhibitory circuitries, as shown, for example, by a study with trimethyltin (*20*). Primary cultures of cortical, cerebellar. or hippocampal neurons are being widely used to study mechanisms of excitotoxicity (*16*); cultures of Schwann cells may represent a good model to study the effect of chemicals on myelination. *In vitro* test systems are amenable to biochemical, electrophysiologic, and morphologic examinations. Molecular biology approaches allow the study of toxicants on gene expression; techniques such as transfection or the use of antisense oligonucleotides allow the manipulation of cells to address specific questions on the role of certain genes, and proteins, in neurotoxicity. The same caution as for any *in vitro* test system, particularly when the results are extrapolated to an *in vivo* situation, also applies to mechanistic studies. For example, do the results obtained with a cell line stably transfected with a certain gene, apply to a different cell type where the gene is expressed constitutively, perhaps to a lesser degree? How does a phenomenon such as neurite outgrowth differ when investigated *in vitro* in pure neuronal populations, from the *in vivo* situation, where glial cells may directly and indirectly play a most relevant role? Despite these potential problems, *in vitro* systems are extremely relevant from the point of view of mechanistic neurotoxicology, and their use is expected to increase.

In Vitro Systems for Neurotoxicity Screening

A goal of *in vitro* systems is that of providing a quick and inexpensive way for assessing potential neurotoxicity. Several approaches have been proposed to identify neurotoxicants and to distinguish them from cytotoxicants, as well as to rank neurotoxicants for toxic potency (*11,13*). Screening is by definition a first-tier evaluation of chemicals that will be followed by additional more specific and complex tests. The same criteria for *in vitro* screening approaches for other end points of toxicity also apply to the neurotoxicity screening; they are *a*) low incidence of false negatives and false positives; *b*) high correlation with *in vivo* toxicity data; and *c*) sensitive, simple, rapid, economical, and versatile. As indicated by many scientists, the challenge in developing *in vitro* systems for neurotoxicity is that of differentiating cytotoxicants from neurotoxicants. Because any cell culture system poorly mimics the intact nervous system, it is important to utilize a variety of *in vitro* models and a multiplicity of end points to parallel, at least in part, neurotoxicity in the whole animal (*11*). Although the use of a test battery is always advocated and recommended, the choice of the systems to be used has not yet been defined, as various investigators have proposed different though similar approaches (*14,18*). A basic initial test battery should include a neuronal and a glial cell line, possibly of human origin, one nonneuronal cell line, and a slightly more complex system such as the rat primary micromass (*21*) or brain reaggregate cultures (*11*). Recently, *in vitro* systems that could mimic the blood–brain barrier, a potential target for many neurotoxicants, have also been proposed. They consist of cocultures of glial cells and endothelial cells (or canine kidney cells) and have been characterized, but not yet sufficiently tested, for their suitability in detecting neurotoxicants (*22,23*).

End points to be measured in *in vitro* tests should include indicators of cytotoxicity and viability as well as neurotoxicity. Basic tests of cytotoxicity and viability are common to most cell types and include measurements of cell death, membrane permeability, mitochondrial function, cell growth and reproduction, energy regulation, and synthesis of macromolecules. If these end points are affected by a chemical, one cannot conclude that the chemical is neurotoxic, but only that it displays cytotoxicity, whose potency can be readily established by conducting concentration–response experiments. However, the use of nonneuronal cell types may provide initial information on whether the chemical may have differential effects or display different potencies in nerve versus nonnerve cells. For the screening of putative neurotoxicants, neural-specific end points representing neurochemical, neuromorphologic,and neurotransmission functions are necessary (*11,13*). The choice of such end points is not an easy task, as the number of parameters that can be measured can be endless. This issue probably represents the hardest problem to be surmounted in order to implement the use of *in vitro* systems as effective screening and predictive tools. Among the biochemical measurements that have been proposed are assays for neurotransmitter synthesizing enzymes (glutamic acid decarboxylase, dopamine hydroxylase, choline acetyltransferase, tryosine hydroxylase), acetylcholinesterase, neuron-specific enolase, glutamine synthetase, and various neuronal receptors, to mention only a few. Morphologic end points such as neurite extension can also be measured and quantified (*24*). If neurotoxicity end points are affected at concentrations lower than those producing cytotoxic effects, a chemical can be considered a potential neurotoxicant. The concern, however, remains that the neurotoxic end points representing the target for a certain chemical may have been missed by the assays chosen for the neurotoxicity screening, thus generating a false negative result.

A number of considerations should be made with regard to potential problems related to experimental design and technical aspects of *in vitro* neurotoxicity tests, all of which indicate the need for a strict standardization of experimental procedures. For example, the source and passage of the cell line, the cell density, the effect of solvent, the source and composition of the media, and the duration of exposure are all potential sources of error. The fact that several chemicals must be metabolized to active toxicants may be good motivation to include microsomal preparations (the S9 fraction) to the test system, as is routinely done, for example, in the Ames test for mutagenicity. However, even these preparations need to be carefully standardized.

The essential step for any battery of *in vitro* neurotoxicity tests is their validation. This process is of utmost importance for consideration and possible use of *in vitro* tests in the regulatory and risk assessment arena. Validation should establish the sensitivity and specificity of the test battery, its reproducibility among laboratories, and its degree of predictivity of human neurotoxicants. Though a 100% correlation between *in vitro* and *in vivo* end points would be difficult to achieve (as even animal tests are

not 100% predictive of human toxicity), this should be the goal to strive for (*11*), notwithstanding the fact that some *in vivo* end points (e.g., sensory functions, long-term memory) do not have *in vitro* counterparts. The validation process should require the choice of a defined test battery (both in terms of test system and of end points) and the testing, under standardized conditions, of a large number of chemicals, some of which are neurotoxic (albeit with different targets and mechanisms of neurotoxicity) and others that are known not to affect the nervous system. Such a process would require a well-organized, strictly controlled, and carefully managed effort similar to others that have already taken place (for example, the study of *in vitro* alternatives to the Draize test). To date, however, such multicenter effort has not been carried out, and the literature provides only examples of validation efforts in which few chemicals were tested and different test batteries were used (*25,26*). Such studies, though interesting and useful, do not offer sufficient information and confidence for any utilization of their results in the hazard assessment process. They should, however, be an impetus for larger efforts to be initiated.

Future Perspectives

One of the goals of *in vitro* testing for toxicants should be that of reducing the use of animals rather then replacing them for toxicity testing (*27*). As such, *in vitro* tests should complement current *in vivo* tests, as it is unlikely that *in vitro* methodologies will become a complete alternative to existing approaches and testing methods. The efforts to validate a substitute for the Draize test represent a case in point, as *in vitro* alternatives have so far failed to be better or even equal predictors of eye irritation potential as the Draize test itself (*12*). The case of neurotoxicity is as much, if not more, complicated. Major problems related to the development of *in vitro* methods for neurotoxicants are the complexity of the nervous system (both structurally and functionally) and the multiplicity of potential targets.

It is highly unlikely that a single test will be developed for neurotoxicity. The suggested battery of tests offers some promise. However, as a screening device, practicality will necessarily limit the number of end points that can be measured, with the ensuing risk of generating potential false negatives. Before a battery of *in vitro* tests can be taken into serious consideration as an alternative, a large validation study should be carried out; several laboratories from different countries should be involved in the testing of several hundred compounds. Such effort, and the necessary funding, can only come from a common action of European, North American, and other regulatory and scientific agencies, institutions, and foundations. National and international agencies and scientific organizations should promote the formation of a panel of neurotoxicologists and statisticians who would meet, following a series of discussions and perhaps additional pilot studies, to define the nature of the battery (cell types, end points), and oversee the completion of the large validation experiment. Such a validated *in vitro* battery could serve as the basis for prioritization of chemicals for further *in vivo* testing.

In the meantime, *in vitro* approaches can be useful as screening tools for particular situations or classes of compounds. For example, cells expressing acetylcholinesterase or neuropathy target esterase have been used to screen for the inhibitory potency of organophosphorus esters and may be useful as predictors of acute cholinergic toxicity versus delayed neurotoxicity (*28*). As previously mentioned, *in vitro* systems are very useful in studying mechanisms of neurotoxicity, and represent an important component of all stages of toxicity testing.

In summary, it is tempting to conclude that neurotoxicology does not offer much promise in terms of the development of alternatives to the current testing procedures. Certainly, the road may be more difficult than for other, less complex, target organs. However, a large concerted effort to launch the validation process, and to go beyond the necessarily incomplete and fragmented attempts so far, would be of great benefit. With the results of such efforts in hand, the scientific and regulatory communities would be able to decide whether sufficient confidence can be placed in such an alternative approach and to define its role in a concerted testing strategy, or whether *in vitro* tests should be confined for use in specific situations or in mechanistic studies in neurotoxicology.

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