In Vitro Neurotoxicity Testing

The Report and Recommendations of ECVAM Workshop 3\textsuperscript{1,2}

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Preface

This is the report of the third of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM’s main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which a small group of invited experts would review the current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on In Vitro Neurotoxicity Testing was held in Angera, Italy, on 7–11 February 1994, under the chairmanship of Christopher Atterwill. As a result of the discussions which took place, a number of recommendations have been made concerning in vitro neurotoxicity testing, which are outlined in this report.

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\textsuperscript{1}European Centre for the Validation of Alternative Methods. \textsuperscript{2}This document represents the agreed report of the participants as individual scientists.
Introduction

Neurotoxicology, as a recognised scientific discipline, has become more prominent in recent years, both as a basic, mechanistic science and in applied testing, with the introduction of the US Environmental Protection Agency (EPA) guidelines for testing industrial chemicals (2). Due to the complexity and relative inaccessibility of the nervous system, cell cultures have played an extremely important role in research areas such as stroke and neuroprotection, neuroregeneration and neurotrophic factors, and developmental neurobiology. Although the regulatory authorities are aware of these technologies, there have not yet been any recommendations for incorporating alternative models into regulatory neurotoxicity testing, even though many of the accepted in vivo markers (for example, neurotoxic esterase [NTE] and glial fibrillary acidic protein [GFAP]) are present and reactive in in vitro test systems (3, 4). For example, some important developments have been the demonstration of organophosphate (OP)-responsive NTE activity in neuroblastomas (5) and of GFAP activity in central nervous system (CNS) astrocyte cultures (4).

In the first report of the FRAME Toxicity Committee, published in 1983, it was recommended that significant efforts should be made to develop cell culture models for neurotoxicity testing (6). Until then, much of the work had centred on the use of ex vivo and subcellular tissue preparations. Furthermore, in the second report of the FRAME Toxicity Committee (1991) it was suggested that a stepwise in vitro neurotoxicity test battery or batteries should be developed, which should include models of both the CNS and peripheral nervous system (PNS) (7).

Subsequently, various groups have put forward models and systems for pre-validation and validation. In 1989, Atterwill (8) proposed a tiered, stepwise, in vitro CNS testing procedure using cell lines and primary organotypic cultures. Various other models have also been proposed (5, 9) and are being investigated (Table I). In 1992, the US National Research Council put forward a protocol for an in vitro neurotoxicity screening system (10).

However, the most important issue is that of controlled pre-validation and validation of such models, before they can begin to gain industrial and regulatory acceptance. Recently, a multicentre pre-validation study of the first

<table>
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<th>Study (contact)</th>
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<tr>
<td>CellTox/FRAME/ECITTS/ECVAM (Atterwill)</td>
<td>three-tiered hierarchical screen</td>
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<td>BRIDGE/BIO TECH/BIOMED (EU)</td>
<td>mechanistic</td>
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<td>US EPA (Veronesi)</td>
<td>neuroblastoma cell lines</td>
<td>organophosphate-directed neurotoxic esterase inhibition</td>
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<td>Zurich (Bruinink)</td>
<td>chick brain and retina primary monolayer and reaggregate cultures</td>
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<td>NeuroSearch, Denmark (Drejer)</td>
<td>primary central nervous system cultures and PC12 cell lines</td>
<td>biomarkers for membrane potential, Ca$^{2+}$ and cytotoxicity</td>
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</tbody>
</table>

ECITTS = ERGATT/CFN integrated toxicity testing scheme; EPA = Environmental Protection Agency.
tier of the stepwise \textit{in vitro} model proposed by Atterwill (8) has taken place, under the auspices of FRAME and ECVAM, using 43 carefully selected chemicals (11). The results from the first tier phase of this study have been extremely encouraging (4, 12). The current status of the pre-validation exercise (Figure 1) is that five key compounds from the initial 45 investigated are undergoing further testing in the second tier of the model, in other neurotoxicological testing schemes, and in the ERGATT/CFN integrated toxicity testing scheme (ECITTS; 13). In the latter, the toxicokinetic features of the test chemicals will also be taken into consideration. The composite data from these studies will be analysed through ECVAM. The proposals contained in this ECVAM workshop report complement and extend this tiered \textit{in vitro} neurotoxicity testing strategy, and recommendations are made concerning the definitive validation of such a scheme.

\section*{EU Research Activities in \textit{In Vitro} Neurotoxicology}

Research activities relevant to \textit{in vitro} neurotoxicology have been promoted in various EU Research and Technological Development (RTD) Programmes by DGXII (CEC Directorate-General for Science, Research and Development). In the Second Framework Programme (1986–1989), the Biotechnology Action Programme (BAP) contained a pre-normative research sector, which funded six transnational shared-cost contracts (involving 16 laboratories) in \textit{in vitro} pharmacotoxicology, of which two contracts were concerned with neural systems, namely \textit{In Vitro} Screening for Teratogenesis by Anti-convulsants in Neural Primary Cultures and Cell Lines and \textit{The Effect of Neuroactive Drugs on Neuroendocrine Function Using Incubated Hypothalamus and Pituitary Tissues In Vitro}.

In the Third Framework Programme (1990–1993) there were eight shared-cost contracts, involving 40 laboratories arranged into six “European Laboratories Without Walls” (ELWW), in the Biotechnology Research for Innovation, Development and Growth in Europe (BRIDGE) Programme. One contract involved neural systems — \textit{In Vitro} Screening in Neurotoxicology and Pharmacology Using Neural Primary Cultures and Cell Lines. In the Biotechnology (BIOTECH) Programme (1993–1996), one large G (generic) project (\textit{In Vitro Developmental Toxicology} is relevant to neurotoxicology. This comprises five shared-cost contracts, involving 23 laboratories under an overall co-ordinator. The project is complemented by the recent formation of an \textit{In Vitro Testing Industrial Platform} (IVTIP).

The objective of these particular programmes is to encourage and support pre-normative research into the development and evaluation of cell culture systems and into the elucidation of the basic mechanisms of action of compounds, which it is anticipated will lead to the subsequent identification of relevant endpoints for \textit{in vitro} test procedures. The results of these projects are disseminated in peer-reviewed scientific publications, in conference and EU-sponsored reports, and at workshops, as well as in the form of proposals for new \textit{in vitro} models.

Specifically within the BRIDGE Programme, the project entitled Development of \textit{In Vitro} Neural Systems for the Identification of Agents with Toxicological and Pharmacological Potential is a neurotechnological project which provides a multidisciplinary approach, with input from biochemists, pharmacologists, molecular biologists, immunologists and toxicologists. The aim is to develop and pre-validate \textit{in vitro} test systems for agents involved in glutamatergic and \textit{Y}-amino-butytratergic neurotransmission. An \textit{in vitro} test model consisting of cerebral cortical neurons in culture has been used to correlate the cytotoxic actions of a number of excitatory amino acids with their abilities to disturb intracellular calcium homeostasis. This neuronal model is extremely valuable in this context, because it is relatively simple in comparison with the intact brain. An improved, inexpensive and easily manipulated culture system for PC12 cells, which enables the investigation of early-gene expression, has also been developed. Benzodiazepine receptor binding assays employing rodent astrocytes have been assessed for use as a general screen for anti-convulsant activity. However, the lack of correlation between astrocytic benzodiazepine receptor affinities and anti-convulsant potencies invalidates its use as a general screening method. Cultured neurons and brain slices are reported to provide systems for assessing the mechanisms of convulsant toxicity, and can thus be used to identify and screen agents such as polychlorocycloalkane insecticides (cyclocienes and hexachlorocyclohexane isomers; 14).
Figure 1: Status of current multicentre pre-validation study of tiered in vitro neurotoxicity testing scheme

CellTox Centre\(^a\)/Salford University\(^b\)
40–45 chemicals

full prevalidation of Tier I (\& Tier III)\(^c\)
detailed neural cytotoxicological & astrotoxicological in vitro database

CellTox Centre\(^a\)
Tier II reaggregate data

Stockholm University\(^e\)
selected 'key' compounds

specific cellular neurotoxicity data

ECVAM
final procedures & tiered model for full validation

ECVAM
other ECVAM collaborators\(^f\)
additional in vitro/in vivo neurotoxicological & compound-specific information

\(^a\) CellTox Centre, University of Hertfordshire, UK.
\(^b\) Division of Biological Sciences, University of Salford, UK.
\(^c\) see Atterwill et al. (10), Williams et al. (11) and Figure 2.
\(^d\) ERGATT/CFN integrated toxicity testing scheme (ECITTS).
\(^e\) Department of Neurotoxicology and Neurochemistry, University of Stockholm, Sweden.
\(^f\) EPA Laboratories, USA; University of Dublin, Ireland; University of Zurich, Switzerland; Zeneca Central Toxicology Laboratories, UK.
In the Biomedical and Health Research (BIOMED-1; 1991–1994) and the Science and Technology for Environmental Protection (STEP) programmes, various activities relevant to in vitro neurotoxicology have been promoted, including projects based on concerted actions, pilot actions, workshops and conferences. As part of the training programmes associated with these research programmes, post-doctoral fellowships have been awarded for training in in vitro neurotoxicology.

The need for research

Research provides information for the subsequent development of relevant cell culture systems and endpoints for testing purposes. The report of a DGXII-sponsored workshop on research and development of in vitro pharmacotoxicology emphasised that “Strong fundamental research is necessary for the development of alternative systems in pharmacotoxicology” (15). The current relevant knowledge in the field needs to be incorporated into the development of appropriate testing procedures. Furthermore, the report states that:

“This approach is similar to that for the in vitro systems widely used in biomedical research to study basic phenomena without the complexities present in vivo. It is based on fostering the knowledge of the fundamental biological processes of the in vitro model in order to further understand the mechanisms of toxicity or drug action, as well as for studies of drug activity and efficacy, risk assessment, etc. An in vitro model should be analysed and judged on its own characteristics and properties, and not merely as a partial reproduction of an in vivo process. Furthermore, these methods require scientific validation, especially if they are intended for use in safety evaluation or in other premarketing regulatory procedures.”

Research programmes promote technology transfer and increase the pool of trained personnel. The close collaborations eliminate duplication and maximise progress and interaction. Joint meetings, exchange of personnel, techniques, ideas and cell cultures, and an opportunity for multidisciplinary approaches, are major advantages of such programmes. Research contracts support the development of particular concepts in neurotoxicology, such as the development of techniques for the primary culture of neurons and astrocytes, and evaluation of the extent to which the functional properties of these cells reflect similar properties of their in vivo counterparts.

There is pressure to make in vitro studies more relevant by using human-derived cells. However, in vitro studies on human brain present problems of ethics, safety and logistics. A partial solution is to develop and use more appropriate cell lines. Nevertheless, the neonatal rodent primary culture systems of neurons and astrocytes are well-developed and generally accepted systems for studying brain cell development, physical and functional interactions, and chemical-induced toxicity.

Recommendations

1. Research programmes foster collaborative studies, which provide the basic mechanistic information on culture systems and endpoints, and on their relevance to in vitro testing. In neurotoxicology, more mechanistic research is necessary to better define the basis of the complex actions of many neuroactive compounds, so that specific and relevant endpoints can be developed.

2. The development of mechanism-based drugs and other chemicals by industry requires the parallel development of mechanism-based in vitro toxicity tests which cover aspects/endpoints involving receptors, ion channels and gene expression. Attention should be given to the appropriate concentrations at which these chemicals exert their effects.

3. Research projects should elaborate proposals for new in vitro models (for example, those which could be included in the third tier of a hierarchical testing strategy), which could then be considered by ECVAM for inclusion in multicentre pre-validation and/or validation studies.

4. Training in in vitro research provides the in vitro neurotoxicologists of the future and should, therefore, be supported.

Current Regulatory Aspects

Current regulatory and industrial attitudes towards using mechanistically designed in vitro models to parallel conventional neuropathological studies are outlined in this section. Such models should be included at the earliest stage of neurotoxicity assessment, to provide valuable data on neurotoxic mechanisms of action which can then be incorporated into future chemical design, compound selection and risk assessment.
**Present guidelines and the whole-animal approach**

The EPA is aware of the recommendation in 1992 by the US National Research Council Committee on Neurotoxicology and Models for Assessing Risk for New and Existing Chemicals, that "existing in vitro methods be exploited more extensively than at present to identify and analyse the mechanisms of neurotoxic action at cellular levels" (10). However, there are no present or pending guidelines for in vitro neurotoxicity testing. Currently, for regulatory purposes, data derived from in vitro tests cannot stand independently of animal data, but may be used as a supplement to it. In particular, in vitro models may be useful in generating structure-activity relationship (SAR) data. The EPA's rationale for the existing guidelines is based on its perception that most in vitro tests are poorly validated, lack correlation with results from animal procedures, and lack focus.

The current neurotoxicity guidelines which require animal testing provide an integrated index of the neurotoxic potential of a test compound. Animal procedures are advantageous since they provide data on pharmacokinetics, regional neuropathic specificity, neural regeneration, age-related and sex-related neurotoxic effects, etc., which can be interpreted for risk assessment purposes. Within the guidelines, primary evaluation combines neuropathological and functional testing parameters. These can be supplemented, when required, by electrophysiology, more elaborate behavioural tests and, in certain cases (for example, for agrochemicals), esterase inhibition data. Tests conducted according to the existing neuropathology guidelines can distinguish between a neurotoxic and a non-neurotoxic compound, CNS and PNS damage, regional brain specificity, and cellular targets of neurotoxic damage.

However, in spite of its ability to predict neurotoxicity, there are a number of shortfalls in the current whole-animal approach to evaluating the safety of chemicals. These include escalating costs, slow throughput of compounds, and increasing animal usage. In addition, the tests required by the present guidelines do not generate mechanistic data on the chemicals investigated. Thus, it is proposed that judiciously selected in vitro models are employed as mechanistic adjuncts for describing neuropathic damage. Such a combined in vitro/in vivo experimental design will provide a more extensive characterisation of the possible neurotoxic effects of a particular chemical, and will optimise the interpretability and utilisation of such data for future chemical design.

**Concerns about the present regulatory guidelines**

The inflexibility of the current guidelines does not permit useful and focused neurotoxicity data to be generated in the most rational way. The relevance of certain components of the present tests can be questioned on scientific grounds and, in terms of logistics, the use of animal models in prescreening for neurotoxicity requires that large amounts of new compounds are synthesised. There are reservations concerning the extrapolation of animal data to the human at risk population. In other areas of toxicology, human cells are often used to improve this extrapolation (for example, data obtained using human hepatocytes may be incorporated into physiologically based pharmacokinetic models, and human cells are used in genotoxicity testing).

The animal procedures included in the present guidelines for neurotoxicity testing cannot be used to fully explore the vast range of new chemicals and their congeners because of the time and costs involved. This means that potentially useful products for society are lost, limiting small company growth and hampering research and development in major corporations.

**Recommendations**

1. The concerns outlined above may be addressed by judicious selection and careful use of the available, scientifically validated, in vitro models in parallel with animal tests (to provide neuropathic data). Such in vitro models should include cellular and subcellular endpoints (for example, cytotoxicological, morphological, biochemical and electrophysiological) which address mechanisms of neurotoxic action. Many of these models have been described in the mainstream toxicology literature and employ endpoints relevant to in vivo lesions of neurotoxicity. The in vitro models are not meant to supplant existing tests, but to enable and direct more focused in vivo investigations.

2. These in vitro models have the ability to enhance the risk assessment process by...
including human-derived cells and tissues in addition to those derived from conventional species of experimental animals, thereby enabling interspecies comparisons to be undertaken.

3. Incorporation of in vitro mechanistic models at an early stage of chemical assessment will greatly reduce the time and costs involved in this testing, will enhance the description and prediction of chemical-induced neurotoxicity, and will aid compound selection.

4. Due to the dire consequences of chemical-induced effects on the nervous system, experimental models which can be used to address putative neurotoxicity in a timely and cost-effective manner are of paramount importance. A combined in vitro/in vivo assessment of neuropathic damage is a much-needed approach to generate a more intelligible, mechanistic description of chemical-induced toxicity and, as such, should be supported by both new and redirected resources. This should be an area of high priority for ECVAM.

Development of New and Improved In Vitro Models and Endpoints

In recent years, many in vitro neurotoxicity test systems, employing a broad spectrum of endpoints, have been developed. Each model has its limitations, which are defined, at least in part, by the test system itself, i.e. by the cells used (primary cell cultures or cell lines, enriched cultures or co-cultures, and tissue or tissue region from which the cells are taken); by the treatment regimen; and by the extent to which reference cell cultures are used to discriminate between true neurotoxicity and general cytotoxic effects. Furthermore, the treatment period and culture conditions (for example, the presence of serum and/or physiological transport molecules, such as transferrin and caeruloplasmin in the case of metal ions, and lipoproteins in the case of lipophilic chemicals) may markedly influence the results and determine the sensitivity of the assay.

For chemicals which require biotransformation (for example, parathion), the presence of cells which are able to metabolise the compound in a manner comparable to that in humans is essential. In addition, indirect effects, such as those of the blood–brain and blood–retina barriers on bioavailability, are often not taken into consideration, although they may greatly determine the toxic effects seen in vivo. In order to understand the criteria which must be satisfied with respect to the development and standardisation of an appropriate neurotoxicity screening system, in which general cytotoxic effects, aspects of nerve-specific and glial-specific toxicity (cell viability and differentiation), and regional-selective and species-selective effects are addressed, an overview of the type of possible effects and related endpoints is given below. Since the endpoints differ depending on the degree of specificity of the effects observed, a division is made between general and specific effects.

General effects

In the absence of information on the toxicological properties of the compound, the neurotoxic hazard may be estimated by using general, non-selective, neurotoxic endpoints. For rapid screening such endpoints should be easy to determine, since the objectives are to obtain an initial indication of the possibility for any chemical-induced neurotoxic effects, and to determine which more detailed, mechanistic, investigations would be appropriate. The endpoints which are currently used for quantifying general effects are summarised in Table II.

General indicators of neurotoxicity include those related to cell survival (necrosis and apoptosis) and to the functions of neuronal and glial cells (proliferation, differentiation and cellular homeostasis). Necrosis is always associated with a decrease in cell viability, as measured, for example, using neutral red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or fluorescein diacetate. Apoptosis, which is characterised by a decrease in cell size and the fragmentation of nuclear DNA, is a normal feature of regulatory processes like development, ageing and tumour suppression. Early apoptotic changes need not be associated with changes in the viabilities of cells, as typically assessed using vital dyes.

Apart from inducing and/or enhancing irreversible changes, such as necrosis and apoptosis, neurotoxins may affect cell proliferation and differentiation. These effects, which may be reversible, are quantifiable by
Table II: Endpoints used in *in vitro* neurotoxicity testing

<table>
<thead>
<tr>
<th>Effect</th>
<th>Assay</th>
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<tr>
<td><strong>General endpoints</strong></td>
<td></td>
</tr>
<tr>
<td>Cell necrosis</td>
<td>neutral red uptake, MTT reduction, fluorescein diacetate hydrolysis, lactate dehydrogenase leakage, ethidium bromide</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>ELISA* for determination of nucleosomes, DNA fragments, and nick and labelled DNA</td>
</tr>
<tr>
<td>Proliferation</td>
<td>cell counting, [³H]-thymidine incorporation, flow cytometry</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
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<tr>
<td>Glia</td>
<td>glial fibrillary acidic protein, monoamine oxidase B, myelin basic protein</td>
</tr>
<tr>
<td>Neurons</td>
<td>transmitter metabolism, uptake and content, microtubule-associated proteins</td>
</tr>
<tr>
<td>Cell homeostasis</td>
<td>voltage-selective and ion-selective fluorescent dyes</td>
</tr>
<tr>
<td><strong>Specific endpoints</strong></td>
<td></td>
</tr>
<tr>
<td>Receptors</td>
<td>radioligand binding, electrophysiology, dye measurement (Fluo-3, Fura-2), cyclic nucleotides (RIA&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Ionotropic</td>
<td>inositol phosphates (radio-labelled or mass measurement), intracellular pH (cytosensor)</td>
</tr>
<tr>
<td>Metabotropic</td>
<td></td>
</tr>
<tr>
<td>Ion channels</td>
<td>electrophysiology, ion fluxes&lt;sup&gt;[³⁶Rb, ³²Na, ⁴⁵Ca, ³⁶Cl]&lt;/sup&gt;, dye measurements</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>protein phosphorylation ([³²P]ATP incorporation, blotting)</td>
</tr>
<tr>
<td>Enzymes</td>
<td>acetylcholinesterase, choline acetyltransferase, monoamine oxidase, neurotoxic esterase</td>
</tr>
<tr>
<td>Uptake systems</td>
<td>radio-labelled ligand uptake</td>
</tr>
<tr>
<td>Release</td>
<td>radio-labelled tracers, endogenous release (HPLC-ECD&lt;sup&gt;c&lt;/sup&gt;, RIA&lt;sup&gt;b&lt;/sup&gt;, bioassays for cytokines), electrocapacitance</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>ATP levels (luciferin/luciferase assay; HPLC)</td>
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*enzyme-linked immunoabsorbent assay.*

*radioimmunoassay.*

*high performance liquid chromatography — electrochemical detection.*
determining the cell count, \[^{3}H\]-thymidine incorporation, cytoskeletal markers (for example, GFAP, neurofilament protein [NFP]), and neurite outgrowth, and by measuring various biochemical markers of differentiation. At a more subtle level of cellular function, the effects of neurotoxins on cellular homeostasis (for example, membrane potentials, intracellular ion concentrations and pH) are readily quantified using fluorescent dyes developed specifically for these purposes. However, minor effects on cellular homeostasis need not result in prolonged changes in cellular function and, therefore, they require further investigation to establish potential alterations in the abilities of the cells to respond to physiological stimulation. For the more general endpoints, it may be necessary to demonstrate the neuronal (rather than glial) specificity of the toxic effects.

**Specific effects**

In some cases, the mechanism of action of a potential neurotoxin is known or suspected. For example, neurotoxicity may be mediated by an acute interaction with neurotransmitter/hormone receptors, ion channels, uptake/release systems or intracellular enzymes. These effects may be measured specifically in brain homogenates, primary neuronal cultures or neuronal-like cell lines. Techniques which may be used to evaluate neurotoxic effects at specific targets are given in Table II. The model system used should be well-characterised with respect to the presence of receptor subtypes, isoenzymes, etc. which are relevant for the specific neurotoxin being studied.

**Pharmacokinetic modelling**

A number of in vitro and animal methods can provide valuable data for prediction of the in vivo pharmacokinetics of chemicals. Physico-chemical properties, such as lipophilicity, molecular size, ionisation and protein binding, are important for predicting the compound's reactivity and its ability to cross biological membranes. These data can form the basis for developing computer models, which may, for example, describe the passage of a particular chemical across the blood-brain barrier and its accumulation in the brain, if the physiological parameters influencing the behaviour of the compound are known.

**Novel endpoints and future research needs**

Some important issues, which are uniquely related to the nervous system, await the further development of basic methodology before they can be applied to in vitro testing strategies. For example, the detection of chemicals which produce fibre demyelination requires the development of tests which involve complex co-cultures and reaggregate cell cultures, since the interaction between, for instance, Schwann cells and contacting axons is necessary for myelin production; the effects of chemicals on the latter could then be assessed using morphological and biochemical markers. Studies of axonal degeneration due to neurotoxin-induced disruption of cytoskeletal components will also benefit from a better understanding of the requirements for culturing mixed populations of cells.

Axonopathies resulting from the disruption of microtubule-mediated transport mechanisms also need to be considered. The responses of glial cells, and an endpoint such as elevated GFAP levels in reactive astrocytes, are likely to prove useful for monitoring astrototoxicity. Age-related effects, including increases in lipid peroxidation and decreases in mitochondrial activity, need to be studied further, as do changes in the accumulations of tau protein and cytoplasmic inclusions. The means by which disturbances of basic neuronal functions can lead to altered gene expression and plasticity are also likely to be understood more clearly in the future.

It is probable that cell cultures derived from the adult PNS will find applications in routine testing sooner than those from the CNS, although measurements of glutamate activity in spinal cord explants may be an exception. “Designer” human cells, produced by the immortalisation of normal cells, are likely to be an important future development.

**Validation of In Vitro Models**

It is considered that, in the first instance, the tests put forward for validation should be simple and should be designed to detect a broad array of chemical insults to the nervous system (i.e. industrial and agricultural chemicals, and pharmaceuticals). The models should be able to detect compounds which may be toxic to either the adult or developing CNS and PNS, since they are intended for use as prescreens, and to act as an interface
between compound discovery and regulatory development in the industrial setting. Such a model would also be potentially useful as an adjunct test in regulatory toxicity testing. A tiered approach (Figure 2) was the option preferred by the workshop participants, on the basis that it would give an optimum balance between the accurate detection of possible neurotoxins combined with ease, speed and resource efficiency. The progression of a compound through the tiers is considered to be discretionary.

In the first tier, potential toxicity should be assessed in primary cultures which are enriched separately in neurons and astrocytes derived from cells isolated from whole brain. To screen for agents which specifically affect the PNS, primary cultures of dorsal root ganglia should be included in the repertoire of test systems to be employed in the first tier. The endpoints measured should include MTT reduction, neutral red uptake and fluorescein diacetate hydrolysis. In addition, the expression of cell-specific markers, such as GFAP and NFP, should be determined. Where relevant, specific endpoints should be employed (for example, when testing organophosphates). Tests with clonal cell lines of neural origin should also be included in this first tier. These cells should exhibit low stringency culture requirements, a potential to differentiate, and a rapid doubling time. The determination of growth, proliferation, motility, adhesion and process formation in these cells will serve as a first screen for chemical-induced developmental effects.

The second tier of the proposed scheme should involve screening of those compounds found to be positive in the first tier tests in whole-brain reaggregate cultures. In addition, clonal cell lines of neural origin (including human-derived cells) and high culture stringency, should be used to determine the specific effects of the putative toxin on defined neural functions (for example, neurite properties). The endpoints to be evaluated in this tier should include GFAP, NFP and myelin basic protein, and the tier should include the malondialdehyde assay for lipid peroxidation. A simple indicator of cytotoxicity should also be included. Furthermore, basal and induced levels of calcium ions should be measured in a human cell line.

It is recommended that persistently positive compounds are studied further. Investigations of their basic mechanisms of neurotoxicity, using appropriate state-of-the-art end-points, should be conducted. These could be considered to be in a third tier of the model, which awaits further definition.

The tiered testing scheme proposed in Figure 2 cannot detect the effects of active metabolites which are formed outside the nervous system. The co-culture of neural cells with hepatocytes, or the use of S9 fractions, are not considered to be desirable. This was identified as an important area of pre-normative research. Similarly, in vitro blood–brain barrier models require further development and cannot be included in the testing scheme at present. Information about the penetration of compounds into the brain may be obtained from physicochemical computer-based models and in vivo pharmacokinetic studies.

Recommendations for specific validation criteria

1. In the first instance, the set of test compounds should be selected by the organisation overseeing the validation study, and should include five neurotoxic and five non-neurotoxic agents, all of which are readily soluble.

2. Ideally, the validation of in vitro tests should be carried out in parallel with an in vivo study conducted according to current regulatory guidelines.

3. Defined protocols should be established for the culture of specific cell types, and for all other experimental procedures. All participating laboratories must adhere strictly to these protocols.

4. Cell lines of neural origin and high culture stringency could include PC12, SH-SY-5Y and IMR32. Cell lines with low culture stringency could include C6, 138MG and D384 gliomas.

5. Two or three independent groups, having the proven ability to perform the tests which are to be validated, should be involved in the interlaboratory study.

Conclusions and Recommendations

In view of the consequences of chemical-induced damage to the CNS and PNS, alternative experimental models for investigating putative neurotoxicity in a timely and cost-effective manner are required. It is considered that a combined in vitro/in vivo assessment
Figure 2: Tiered scheme for *in vitro* neurotoxicity testing

**Tier I**

<table>
<thead>
<tr>
<th>Primary</th>
<th>Clonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>- whole-brain astrocyte-enriched and neuron-enriched cultures</td>
<td>- low culture stringency</td>
</tr>
<tr>
<td>- dorsal root ganglia cultures</td>
<td>- potential for differentiation</td>
</tr>
<tr>
<td></td>
<td>- rapid doubling time</td>
</tr>
</tbody>
</table>

**Tier II**

- MTT reduction
- fluorescein diacetate hydrolysis
- neutral red uptake

- glial fibrillary acidic protein
- neurofilament protein
- (neurotoxic esterase/acetylcholinesterase)
- direct cell counting

**Tier III**

<table>
<thead>
<tr>
<th>Primary</th>
<th>Clonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>- whole brain reaggregate cultures</td>
<td>- human/animal</td>
</tr>
<tr>
<td></td>
<td>- high culture stringency</td>
</tr>
<tr>
<td></td>
<td>- high differentiation potential</td>
</tr>
</tbody>
</table>

- glial fibrillary acidic protein
- neurofilament protein
- malondialdehyde
- myelin basic protein
- cytotoxicity
- $\text{Ca}^{2+}$ levels

- basic mechanisms
- new models and endpoints
of neuropathic damage is now needed, to generate a more intelligible and mechanistic description of neurotoxicity. Current EU activities in neurotoxicology and neuropharmacology, supported by DGXII and ECVAM, should foster collaborative research and support training in *in vitro* research methods, to provide future innovation in *in vitro* neurotoxicology.

Endpoints of neurotoxicological damage which relate uniquely to specific components of the nervous system await further development before they can be applied routinely to *in vitro* testing strategies. Examples of these include nerve fibre demyelination and effects on microtubular and cytoskeletal components related to axonopathies. Reactive astrogliosis, involving elevated GFAP levels, is seen as an important response which can be detected *in vitro*. The production of "designer" transfected and/or immortalised human neural cells is likely to be an important advance in the future, for example as a means to incorporate metabolising enzymes and receptors in cells. In addition, an awareness and understanding of how disturbances in basic neural functions lead to altered gene expression and plasticity will have an important impact upon the development of appropriate strategies for *in vitro* neurotoxicity testing.

In order to detect a broad range of chemical insults to the CNS and PNS, an *in vitro* tiered testing scheme (Figure 2) has been proposed as the preferred option for validation. This model, which should be used in a sequential, stepwise manner, was felt to give an optimum combination of the accurate detection of neurotoxic effects with ease, speed and resource efficiency. The first tier comprises tests with primary CNS-derived cultures enriched in neurons or astroglial cells from rat whole-brain, tests with primary cultures of dorsal root ganglia for detecting peripheral neurotoxins, and tests with clonal cell lines of neural origin. Endpoints in this tier include those for cytotoxicity and for neural cell-specific markers (GFAP and NFP), as well as more specific endpoints where relevant (for example, acetylcholinesterase and NTE).

The second tier comprises tests using rat whole-brain reaggregate cultures, incorporating a range of neural cell-specific endpoints in addition to indicators of general cytotoxicity. The third tier, consisting of tests to be undertaken with chemicals which are positive in the first and second tiers, awaits further definition and method development, but is envisaged to include state-of-the-art endpoints for mechanistic neurotoxicological phenomena. At present, the inclusion of metabolising systems in any tier cannot be recommended, but the development of such tests was identified as an important area of research. Similarly, efforts should be directed towards the development of cellular blood–brain barrier models, utilising organotypic models of this type. The third tier can be more flexible, but it is envisaged that it would include models such as the *in vitro* PNS and CNS mast cell preparations for studying aspects of neuroimmunotoxicological insult (16).

It is recommended that the composite tiered testing model outlined in Figure 2 should be subjected to full validation under the auspices of ECVAM. If successfully validated, the model should be considered for use, firstly as a neurotoxicological prescreen in the industrial setting, and then as a possible adjunct for regulatory testing purposes. When trying to evaluate the hazard of potential neurotoxins, the likelihood of a compound reaching the CNS must be taken into account. The characteristics of the blood–brain barrier and the physicochemical properties of the test chemical will ultimately determine the uptake and accumulation of the chemical in the brain. Data from computer models for predicting passage across the blood–brain barrier should therefore be incorporated into the hazard evaluation process.

The major recommendations from the ECVAM workshop on *in vitro* neurotoxicity testing are summarised below:

1. To develop and evaluate alternative experimental models and endpoints relevant to the human paradigm for investigating putative neurotoxicity in a timely and cost-effective manner.

2. To adopt a combined *in vitro/in vivo* approach for the assessment of neuropathic damage.

3. To foster collaboration among the various EU neuropharmacotoxicological research initiatives, to support training in *in vitro* methodology, and to develop new and specific biomarkers, endpoints and models for *in vitro* neurotoxicological studies.

4. To devise and validate a three-tiered *in vitro* model, encompassing basal cytotoxic, cell physiological and neural cell-specific endpoints, for studying CNS
and PNS neurotoxic insult. This tiered model will include primary and secondary neural cell cultures of animal and human origins, as well as organotypic whole-brain cultures.

5. To validate such a tiered testing scheme on a multicentre basis, under the auspices of ECVAM and using an agreed set of test compounds. The validation process should be undertaken in parallel with in vitro neuropathological and neurotoxicological studies on key compounds.

6. To use toxicokinetic data to modify in vitro neurotoxic critical concentrations in the final comparison with critical in vivo concentrations.

References