Preface

Neurotoxicity assessment with in vitro systems is the focus of both increasing expectations and heightened challenges. Such systems prospectively offer a means to improve screening efficiency for potential neurotoxicants, a method for better understanding mechanisms of toxicant action, a decreasing use of animals, and a means to obtain data from human samples. On the other hand, in vitro systems have not yet been used in consistent, broadly applied formats that would validate and exploit their value for neurotoxicity testing. Inherent problems, such as test chemical concentration and delivery, lack of heterogeneous cell–cell interactions, immaturity of cell types available, phenotypic variations induced by culture techniques, and insensitivity of endpoints tested, significantly impede the use and interpretation of in vitro assays. In addition, standardized metrics and methods for comparing results across studies and laboratories, as well as benchmark criteria for linking in vitro to in vivo studies, are often lacking.

The purpose of *In Vitro Neurotoxicology: Principles and Challenges* is to synthesize principles and concepts of in vitro neurotoxicology that will facilitate the development of significantly improved methods and systems for in vitro neurotoxicity testing, with emphasis on their relevance to in vivo systems. An outstanding list of contributors has been assembled, including well-respected leaders in the field and new investigators who are exploring emerging frontiers in the area of genomic toxicology. Contributors have taken a fresh look at their own and others' work, critically and comparatively analyzed it across experimental systems and toxicants, and formalized essential principles for in vitro neurotoxicity testing. In most cases, chapters are arranged around major themes or central ideas, rather than around individual toxicants or specific in vitro models. Most chapters are collaborative efforts that address a theme and employ examples comprised of multiple experimental systems and endpoints. The chapters emphasize several neurotoxicants that are of prominent human health concern and about which metabolism and dose–responses are best understood, both in vivo and in vitro: lead, mercury, organophosphorus insecticides, polychlorinated biphenyls and dioxin, ethanol, and endogenous proteins.

There are already several excellent articles and monographs that describe materials and techniques applicable to in vitro neurotoxicology, such as cell lines, methods of primary cell culture, brain slice preparations, and in vitro

assays for viability and function. Rather than repeating the contents of these previous works, *In Vitro Neurotoxicology: Principles and Challenges* provides an Appendix containing a critically reviewed list of related works. The list, carefully selected and annotated by the contributors, includes important review articles, books on in vitro toxicology, neurotoxicology, and in vitro neurotoxicology, and chapters from methods manuals. The Appendix collects in one place references to most of the major reviews and seminal work related to in vitro neurotoxicology that have appeared in the past ten years.

Evelyn Tiffany-Castiglioni

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Predictive Value of In Vitro Systems for Neurotoxicity Risk Assessment

Marion Ehrich and David C. Dorman

1. INTRODUCTION: NEUROTOXICITY RISK ASSESSMENT

Risk assessment has been broadly defined as the characterization of the adverse health effects of human exposures to environmental hazards and can be divided into four major steps: hazard identification, dose–response assessment, exposure assessment, and risk characterization *(1)*. Hazard identification is defined as determining whether human exposure to an agent can cause an increased incidence of an adverse health effect (e.g., neurotoxicity). Dose–response assessment is the process of characterizing the relationship between the administered or effective dose of an agent and the incidence of an adverse health effect in exposed populations, estimating the incidence of the effect as a function of human exposure to the agent. A dose–response assessment should account for exposure intensity and duration, developmental age, and other factors that may modify the response (e.g., gender, diet). Exposure assessment is the process of measuring or estimating the intensity, frequency, and duration of human exposure to an agent found in the environment or an agent that may be released into the environment. Risk characterization integrates these preceding steps by estimating the incidence of a health effect under various conditions of human exposure.

These four steps form the basis of risk assessment. They are independent of the nature of the adverse health effect (e.g., neurotoxicity vs carcinogenesis), although underlying assumptions (e.g., threshold vs nonthreshold effects) may influence the approaches used.

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Neurotoxicity is defined as any adverse effect on the chemistry, structure or function of the nervous system during development or at maturity induced by chemical or physical influences *(2)*. For a chemical to be regarded as a neurotoxicant, effects on the nervous system should be direct rather than indirect, adverse rather than adaptive, and toxicological rather than pharmacological. Chemically induced neurotoxic effects are of special concern because neurotoxicological syndromes may be delayed and are often progressive or irreversible and prevention is far less costly than treatment *(3,4)*. Only recently have regulatory agencies focused their attention on developing guidelines for the conduct of neurotoxicity risk assessments *(5)*.

Requirements for animal testing of pesticides and some commercial chemicals for neurotoxicity are promulgated worldwide by a number of regulatory agencies. For example, the US Environmental Protection Agency administers the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA). Testing for specific end points indicative of neurotoxicity may also be recommended by the Food and Drug Administration for certain food additives that demonstrate positive results in a very basic, initial neurotoxicity screen *(6)*. It is estimated that only a small fraction of the 70,000 chemicals currently in commerce have been adequately assessed for neurotoxicity *(7)*; thus, there is a need to develop cost-effective screens to assess chemicals for potential neurotoxicity.

Bioassays remain the principal method to identify possible human health risks posed by exposure to chemicals and other potential neurotoxicants. The primary advantage of using animals for hazard identification and risk characterization is that all potential targets for injury (e.g., the many types of cell, tissue, neurochemical) are included in the test system *(4,8)*. This is especially important because neurotoxicants can affect a variety of different organs and tissues and they can induce alterations in chemistry, function, structure, or behavior (*see* Fig. 1). End points of interest in bioassays often include histopathology to assess morphologic damage and batteries of functional, neurobehavioral, neurochemical, and neurophysiological tests to examine the operational integrity of the nervous system *(9)*. Because there are physiological and anatomical similarities among mammals, the finding of a positive response in vivo is taken as evidence that an agent may also pose a risk for exposed humans. This integrated in vivo approach is valuable for a detailed characterization of both the effects and possible mechanisms of suspected neurotoxicants under specific exposure conditions. In vivo methods are relatively well developed and the data are used to determine no observable adverse effect levels (NOAELs), uncertainty factors, and benchmark doses.

The use of animals in toxicity testing is often the subject of intense scrutiny and criticism by the general public. The toxicology community continually

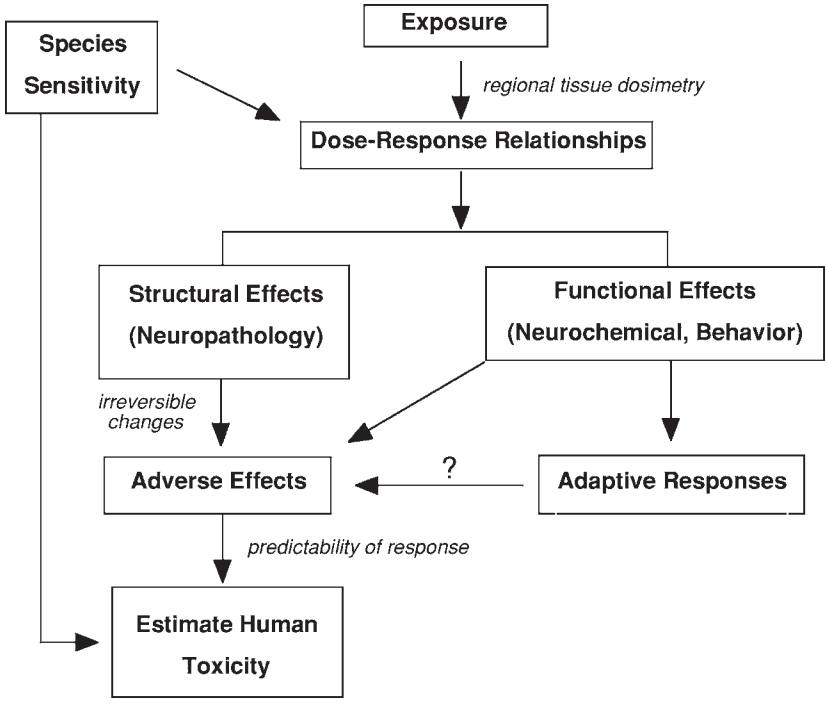

Fig. 1. Critical elements in characterizing neurotoxicity risk from exposure to a chemical. (Reprinted with permission from ref. *9*.)

strives to replace the use of animals in research, to reduce the numbers of animals to the minimum necessary to obtain valid results where replacement was impossible, and to refine all experimental procedures to minimize adverse effects on animals. The use of in vitro test systems is a logical alternative to the use of animals and can often complement and enhance in vivo data. In vitro tests can be sensitive, replicable, valid, and cost-effective. They are amenable to studies done over a wide range of concentrations and over multiple periods of time. Exposure to the test chemical can be tightly controlled and human materials can be used. In vitro data can provide important structure–activity data concerning the relative potency of different chemicals and contribute to choices for chemicals to study in vivo. In addition, in vitro data can be used to identify the mode of action of a chemical and identify critical factors that determine species- or tissue-specific differences in response. Studies of relationships between in vitro and in vivo data have helped to identify these and other factors that contribute to and modify toxicity *(2–4,7,8,10–14)*. It is well recognized that the risk assessment process is improved if data concerning the chemical's mechanism(s) of action are included. However, it

remains speculative whether in vitro data can replace any in vivo data used for the risk assessment of a neurotoxicant. Indeed, in vitro data are rarely considered in the risk assessment process because current statutory guidelines do not classify changes observed in vitro as indicative of an adverse response *(7,10,11,15–17)*.

As with any technology, in vitro systems have distinct limitations. In vitro test systems have a reduced cellular complexity, therefore, the responses observed in vitro may not be representative of those observed in the substantially more complex intact nervous system (*see* Fig. 2). One important structural difference of most neural cell culture systems is the lack of a functional equivalent to the so-called blood–brain barrier. This blood–brain barrier excludes the movement of certain chemicals or their metabolites into the intact nervous system, thereby attenuating the observed neurotoxic response. Additionally, isolated in vitro systems often lack the hepatic and extrahepatic metabolic systems that are normally present in the intact animal to activate or detoxify the agent under investigation. Thus, in vitro systems have only a limited capacity to metabolize selective toxicants. Current choices are often between simple systems that are significantly divergent to the in vivo situation but are easy to manipulate and complex systems that are technically difficult to establish and use. In spite of these limitations, in vitro models are proving useful for the screening of chemicals for neurotoxic potential. In vitro model systems may provide an economical first-tier evaluation that will help to guide more extensive whole-animal studies.

2. SYSTEMS FOR EVALUATION OF NEUROTOXICITY IN VITRO: MECHANISTIC MODELS AND SCREENING TESTS

A number of reports suggest that in vitro tests for neurotoxicity would be more useful in providing mechanistic information than they would be for general screening purposes for agents of unknown toxicity. This suggestion is based on the complexity and multiple targets of the nervous system and the comparative simplicity of in vitro test systems *(2–4,8,18)*. Although the development of screening tests may appear formidable to some, the suggestion has been made that end points indicative of cytotoxicity (i.e., cell viability) could be more sensitive in cells of the nervous system exposed to neurotoxicants than cells of extraneural origin. This assumption, based on the premise of tissue selectivity, may have value when appropriate comparisons are made. However, although potent cytotoxins can be neurotoxicants, they are likely to be toxic to other tissues as well (e.g., liver, kidney, lung) *(2,12,15,16)*.

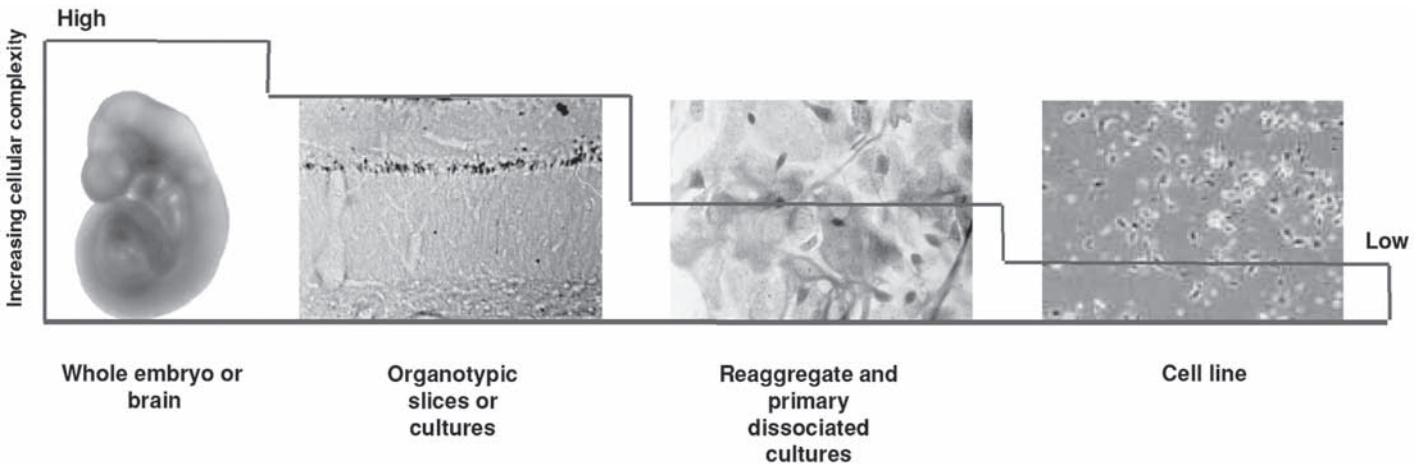

Fig. 2. Types of in vitro systems used in neurotoxicology research. Cultures composed of individual cell lines have the least structural complexity and predictability to man.

Like their in vivo counterparts, in vitro neurotoxicity screens require the use of test systems and end points that are sensitive, efficient, and neural-specific. The systems should provide low numbers of false negatives or positives *(2)*. The sole reliance on a single experimental model, whether it be in vivo or in vitro, with a limited number of biological markers is not generally considered sufficient for estimation of risk. For this reason, a tiered system for in vitro neurotoxicity screening has been proposed *(15)*. The tiered system was designed to include cytotoxicity and cell-specific effects determined in simple and more complex in vitro systems in the first and second tiers and mechanistic studies in the third tier. An initial study, using a neuronal cell line and multiple end points (some neural-specific, some not), suggested that the number of end points was not sufficient to use a single clonal cell line as a screening system for neurotoxicity *(19)*. This contrasts with results of another large study examining cytotoxicity in non-neural cells, which suggested that cell viability appeared to be a valid indicator of general toxicity *(20)*. Regardless of end points, however, concentration–response and time–response studies need to be included into any in vitro test screen *(10,20,21)*. It has also been suggested that in vitro screens should include human neural cells to allow evaluation of interspecies differences in response *(1,3,14,16,18,22)*.

Many neurotoxicants have unknown and/or multiple mechanisms of action *(4,11,23,24)*. The use of in vitro test systems often yields valuable mechanistic data on chemical-induced neurotoxicities and thus offers an attractive alternative to the use of animals for this type of research. Mechanistic studies can be designed to evaluate multiple mechanisms of action and can be modified to the toxic agent of concern. End points can include general glial or neuronal measures, neurotransmitter systems, and indicators of the biochemical and electrical responses of neural cells. Reversibility and irreversibility of effects, protective mechanisms and repair, and ability to affect cell proliferation and differentiation can be determined. Characterization of the cellular and molecular substrates and pathways that follow exposure to neurotoxicants can be evaluated *(2,3,7,10,13,15,24)*. The generation of useful mechanistic data requires realization that the data obtained will not necessarily provide explanations for all manifestations of neurotoxicity seen in man and animals, including behavioral, cognitive, sensory, developmental, or age-related effects. Furthermore, standard protocols that include well-defined culture conditions and means to reduce potential for cell instability need to be followed to permit intralaboratory and interlaboratory validation of results *(2,3,7,10,13,15,22)*.

3. IN VITRO MODELING OF IN VIVO SYSTEMS: DOSE–RESPONSE CONSIDERATIONS

It is well recognized that risk assessment considers dose–response data for an adverse effect. Dose (concentration)–response studies can be done with relative efficiency using in vitro test systems, and test compounds can be applied directly without concern for the pharmacokinetic factors of absorption, distribution, metabolism, and excretion encountered in animals. Isolated test systems are often exposed to concentrations of chemicals that far exceed those achievable in tissues from exposed animals or humans. Interpretation of in vitro studies is aided dramatically by detailed knowledge concerning concentrations of the parent chemical or major metabolites in blood, brain, and other potential target tissues. In some cases (e.g., organophosphate insecticides, polychlorinated biphenyls, and ethanol) effects can occur in vitro at concentrations similar to those observed in vivo *(13,25)*. Effects occurring in vitro at lower concentrations than those achieved in vivo may indicate that the test chemical is metabolized in vivo to a less toxic form, the chemical or the active metabolites are excluded from the nervous system, or that compensatory or repair mechanisms occur that attenuate the toxicity of the chemical observed in vivo. Neurotoxic effects that require in vitro concentrations higher than blood concentrations of intoxicated animals may occur because concentrations in target tissues are higher than concentrations in blood. Concentration differences may also depend on the in vitro system used for testing (e.g., cells of neoplastic origin are notoriously resistant to chemical-induced cytotoxic effects). It is, therefore, important to consider the context in which the in vitro data are collected *(8,13,16,25)*.

A specific example of dosing considerations can be noted with exposure of neuronal cell lines of neoplastic origin to cholinesterase-inhibiting organophosphorus compounds. Inhibition of acetylcholinesterase, which is responsible for clinical signs seen in people and animals, occurs following minutes of exposure to physiological (nanomolar to micromolar) concentrations of these agents in neuroblastoma cells of mouse and human origin, yet cytotoxic and lethal effects require many hours and concentrations of these compounds in millimolar ranges *(25–28)*. Primary cell cultures (e.g., neurons isolated from chick dorsal root ganglia) exposed to these same test agents can demonstrate cytotoxic effects to organophosphorus compounds at micromolar concentrations (Massicotte and Ehrich, unpublished).

As noted in several reports, dose–response data obtained from in vitro studies do not generally consider pharmacokinetic differences between in

vitro and in vivo systems, which can limit the potential for in vitro to in vivo extrapolations *(2,3,7,29)*. This, however, does not totally detract from their usefulness, for another application of in vitro test systems is to examine biological processes that may affect the pharmacokinetics of a chemical. For example, useful data concerning the transport of chemicals into the nervous system can be obtained from in vitro test systems. Studies using isolated primary rat neural cultures have demonstrated that the transferrin receptor plays a critical role in the uptake of aluminum, iron, and other metals *(30,31)*. Experiments conducted using isolated brain microvessels have demonstrated the role of amino acid carriers in the transport of mercury to the central nervous system *(32)*. Other investigators have used brain tissue slices, brain homogenates, and other in vitro test systems to examine the metabolism of *m*-dinitrobenzene *(33)*, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *(34,35)*, and other neurotoxicants. Predictive pharmacokinetic models that include in vitro metabolic data extrapolated to the whole animal are, however, still under development *(3,4,16,29)*.

A significant advantage of in vitro test systems is that concentration–response curves can be readily created and concentrations responsible for 50% effects (EC_{50} values) can be determined and compared *(4,13,16,19,25)*. These comparisons are very useful in considering structure–activity relationships among different chemicals. The best comparisons of EC_{50} values are made when concentration–response curves include several data points, when these curves are parallel, when the end point of interest is specific, and when all data are collected under the same conditions. EC_{50} values can also be used to examine whether tissue or species differences in response to a neurotoxicant occur *(3,19,25,36)*. Care, however, must be taken when EC_{50} values are used to compare sensitivity of different end points, especially when comparisons are between nonspecific end points (e.g., cytotoxicity) and specific targets of particular compounds (e.g., esterase inhibition caused by organophosphates), as mechanisms associated with expression of the end points may differ *(12,13,25)*. Considerable care must be taken when comparing in vitro data (e.g., EC_{50} values) and in vivo data (e.g., LD_{50} values, blood concentrations in intoxicated subjects, behavior of exposed subjects). The differences between in vivo and in vitro test systems are so great that correlation of EC_{50} values and LD_{50} values (or blood concentrations) may have little value. To date, the best correlations have occurred with very potent toxicants *(2,3,16,20,37)*.

4. RECOMMENDATIONS AND CONCLUSIONS

Inclusion of data collected during neurotoxicity testing using in vitro systems in risk assessment mandates that end points be relevant and that in vitro

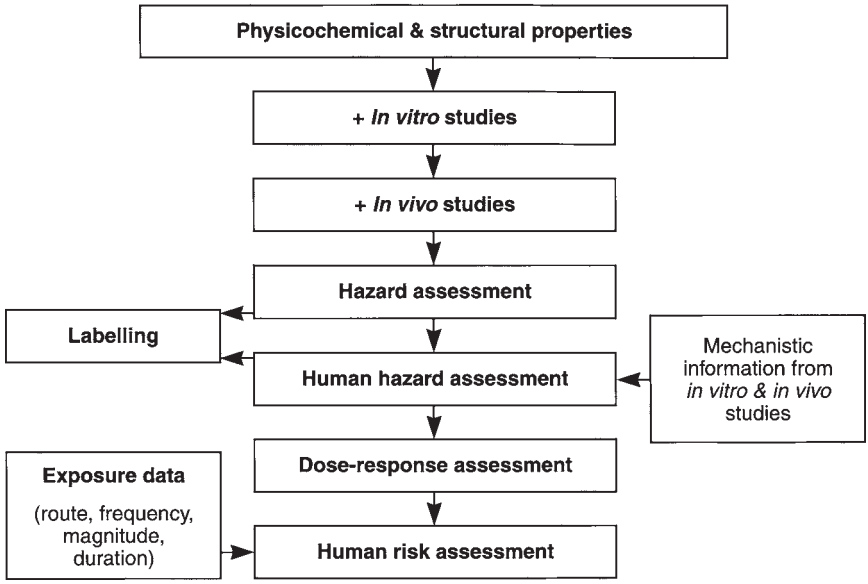

Fig. 3. Scheme for hazard and risk assessment. Reprinted from ref. *11* with permission of *ATLA*.

testing systems be validated. The validation needs to be at multiple test sites and include reproducibility, repeatability among various test sites, protocol standardization, chemical reference standardization, and quality assurance. In vivo methods with reasonably developed in vitro alternatives will be easiest to replace, although it must be recognized that statutory requirements must be met and acceptance may be slow *(2,4,10,11,13,15,16,21,22,25)*. In vitro systems could help classify test chemicals as to their likely mode of action, select chemicals from a larger group for further testing, and suggest which chemicals and which tests should be done in vivo *(8,11)*. In vitro and in vivo tests run in parallel may provide the most information about general toxicity and mechanisms of neurotoxicity, especially for new compounds. In this case, both types of data would be more likely to be included in the risk assessment process *(10,11,13,15)* (*see* Fig. 3).

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