4

Neurotoxicity

Jean Harry, John L. O'Donoghue, and Alan M. Goldberg

ABSTRACT: *Guidelines for testing existing chemicals for neurotoxicity under the Toxic Substances Control Act (TSCA) have been published by the U.S. Environmental Protection Agency (EPA). While the current regulatory process for assessing existing chemicals under TSCA does not use a tier-testing approach, there is widespread support for tier-testing processes. However, there is general disagreement between the Agency and the regulated community over what tests should be used for hazard identification (i. e., first-tier screening test). The regulated community sees the standard toxicity tests which are commonly conducted for systemic toxicity as sufficient for neurotoxicity screening, while the Agency regards its guideline tests as necessary to screen chemicals specifically for neurotoxicity. The guideline tests while frequently referred to as screening tests are expensive and time-consuming and therefore not widely used outside of formal existing chemicals testing programs. Development of true screening tests should be based on a mechanistic understanding of the neurobiological processes which result in neurotoxicity. The most commonly used alternative screening techniques include structure-activity analysis and in vitro methods. In vitro techniques (e.g., primary neuronal cultures, glial cell cultures, organotypic explants) are commonly used today to study mechanisms of neurotoxicity and have the potential for being used for hazard identification. Rapid, inexpensive screening tests would be expected to be useful during the early phases of new product development cycles and thus may have much more pollution prevention potential than existing methods. Such tests may eventually offer methodologies to either replace or complement tests currently used. The complex nature of the nervous system suggests that if in vitro methods gain acceptance as screening tests for neurotoxicity, they will have to be used in batteries of several assays to study multiple endpoints.*

DEFINING NEUROTOXICITY: CONTROVERSIAL BUT CRITICAL

Neurotoxicity is one of several organ-specific endpoints used by regulatory agencies to determine hazards of chemical exposure. Definitions of neurotoxicity have been established by various organizations as the capacity of chemical, biological, or physical agents to cause adverse functional or structural changes in the central or peripheral nervous system **(3, 5, 9,** 10, 15, 16, 18).

In each of these cases, the definition of neurotoxicity is dependent on the controversial interpretation of the word "adverse". Tilson (12) has proposed that the definition of adverse includes alterations from a baseline state that diminishes the ability of an organism to survive, reproduce, or adapt to its environment. It has been suggested that unintended or unwanted effects should also be included under this definition (12). However, such a definition must take into account the possibility that neurobehavioral effects might be produced nonspecifically at high dose levels. Some argue that the definition of neurotoxicity should be defined more in terms of direct nervous system toxicity **(5).**

Clarification of the definition of neurotoxicity is critical to the design of neurotoxicity screening tests, since the designer of screening tests must have a clear understanding of what the testing paradigm is expected to accomplish. For example, tests to detect blurring of vision caused by eye irritation must be designed very differently from those expected to detect vision loss due to methanol intoxication. Interpretation of the results of currently used tests for neurotoxicity can be difficult because the currently used screening tests do not necessarily distinguish between effects which are direct vs. those which are indirect. Direct effects are produced by agents or their metabolizes that produce toxicity

by primarily interacting with target sites in the nervous system. Indirect effects are produced by agents or their metabolizes that produce toxicity by interacting with target sites outside the nervous system. The occurrence of systemic toxicity could complicate the interpretation of functional changes; however, systemic toxicity does not necessarily preclude the use of functional changes in defining neurotoxicity.

A major concern is that nonspecific behavioral changes produced by high-dose level systemic toxicity may be interpreted as providing evidence of neurotoxicity. A well designed neurotoxicity study needs to control for nonspecific toxicity that could impair the assessment of chemically-induced changes in nervous system function. Concerns about indirect effects may be addressed by selecting appropriate dose levels which do not induce a significant degree of general systemic toxicity. In some cases the differentiation between direct and indirect effects may require additional second-tier testing to resolve.

Also of concern is the distinction between effects which are transient vs those which are persistent. Transient effects are those which are considered to be fleeting in time and typically are related to pharmacological processes and the presence of a chemical in the body, while persistent effects have a lifespan which exceeds the lifespan of the chemical in the body. Some transient effects (e.g. seizure activity) are obviously serious, but many others (e.g. changes in enzyme levels or increased rates of whisker twitching) may not have any recognizable consequence, yet in the current risk assessment process, each of these changes could be evaluated as critical endpoints requiring equivalent safety factors.

CURRENT METHODS FOR NEUROTOXICITY TESTING UNDER TSCA

The complexity and integrative nature of the nervous system makes the identification of a single endpoint problematic. As a result, neurotoxic effects are usually measured at multiple levels of nervous system organization, including behavioral, neurophysiological, neurochemical, and neuroanatomical levels. There is general agreement that an assessment of potential neurotoxicity should be based on a number of parameters generated from a variety of tests at relevant dose levels. Historically, morphological methods have been used to detect neurotoxicity; however, assessments of neurotoxic potential can be enhanced by a combination of morphological and functional data. Some neurotoxic agents and pharmacologically active materials (e.g., cholinesterase inhibitors) can cause alterations in the functioning of the nervous system in the absence of morphological changes, thus adding support to an assessment based on different types of endpoints (13).

A number of expert groups has recommended tier-testing strategies for evaluation of chemically-induced neurotoxicity (9, 15). Cage-side observations and the US Environmental Protection Agency (EPA) guideline for a Functional-Observational Battery (FOB) are examples of tests which are considered first-tier tests by the regulated community and the Agency, respectively. The initial phase of a tier-testing strategy is the identification of chemical's capability to produce neurotoxicity at some dose level (i.e., hazard identification). First-tier tests are typified by their capability to assess a large number of animals, usually requiring little or no training of test animals prior to exposure, and generally being relatively simple to perform. The types of observational methods used to detect neurotoxicity (e.g., FOB) have been criticized as labor intensive, subjective, and semi-quantitative. However, the current manner in which clinical signs are collected has also been criticized as being highly variable and poorly documented. Therefore, the development of the FOB has been at least partially driven by efforts to develop methods to place observation of clinical signs under a systematic protocol. Whether first-tier testing is comprised of cageside observations or the FOB, there is widespread agreement that any screening technique should include the following features: 1) the method and endpoints should be

clearly defined, 2) the effects should be quantified using an explicitly stated rating scale, 3) observers should be trained, and 4) a number of endpoints should be assessed to evaluate multiple modalities of nervous system function.

The EPA has considered the inclusion of a quantitative measure of motor activity in the first-tier testing for existing chemicals under Toxic Substances Control Act (TSCA). The Agency's approach on the use of motor activity is based on the large wealth of neurobehavioral pharmacology data using this endpoint. In addition, the fact that motor activity levels can be influenced by the general toxicity of a chemical can be used to aid in the interpretation of observational screening data. However, the use of motor activity as a test for neurotoxicity has been repeatedly rejected by the regulated community which views such tests as having little value for identification of neurotoxicity, prone to interpretation bias, and invalidated as a screening test for neurotoxicity. Other tests which have been included in a first-tier test battery are quantitative measures of limb grip strength and hind limb foot splay. In many situations, functional tests are used in conjunction with other methods including neuropathology.

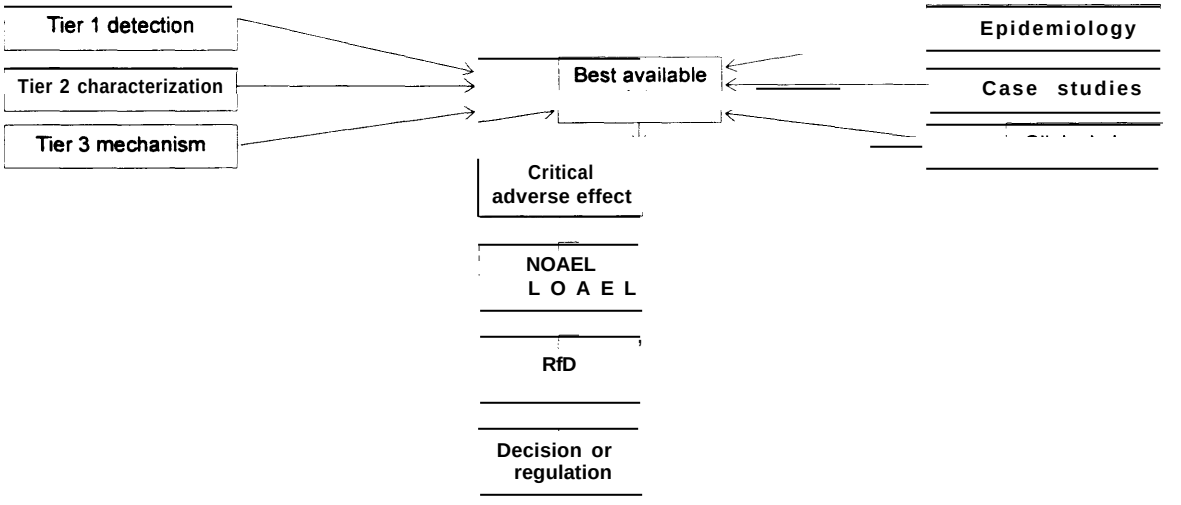
In order to improve identification of agents capable of producing neurotoxic effects, efforts have been made to validate reliable, sensitive measures of neurotoxicity. Increased emphasis on testing for neurotoxicity has been included in the existing chemicals program under TSCA resulting in the development of testing guidelines by the EPA and standardized procedures by the regulated community. Cageside observations for neurological and behavioral changes have been part of toxicological testing practices for many years. The cageside observations and routine pathology studies conducted as part of the data gathering process for systemic toxicity are considered by the regulated community as the first tier for all systemic toxicants, including neurotoxicants. However, given regulatory agency guidelines and the need to provide more quantitative measurements, FOBS have been developed to include more systematic recording of observations. Testing guidelines, such as the FOB, rely on behavioral measures based on the assumption that behavior appears to be the net result of the integrated output of various nervous system processes. A change in such an integrative process could serve as a relatively sensitive indicator of nervous system dysfunction, especially since many of the endpoints assess changes in sensory, motor, and cognitive functions.

Although a number of articles exist in the published literature on the use of observational methods for neurotoxicity testing, not all report equal success in detecting neurotoxic effects, pointing out the need for data on inter- and intralaboratory reliability and interlaboratory sensitivity. The International Programme on Chemical Safety of the World Health Organization is currently sponsoring an international collaborative study on neurobehavioral methods for the FOB, motor activity, and grip strength.

Although observational methods are conceptually the most straightforward, they are also the easiest to confound and can sometimes be difficult to interpret without some internal or external corroboration of results. Given the various biological modalities encompassed in nervous system function and the numerous endpoints used to assess function, questions can arise concerning the significance of a change in a specific endpoint. One of the approaches that has been proposed to deal with such data is to cluster the various observations into functional domains that represent common neurobiological processes (i.e., autonomic function) and generate a composite response score to reflect the functional integrity of a given subset of necrologic processes. This approach would allow data to be evaluated within a small number of neurobiologically meaningful clusters rather than numerous isolated endpoints. While this clustering methodology may be conceptually appealing, widespread acceptance of it will depend on how well the testing community perceives that there is a meaningful biological basis for the clustering.

The second tier of neurotoxicity testing (beyond screening for the potential for neurotoxicity) is generally regarded as providing more

FIGURE 4-1: Use of Data in Regulatory Decision-Making



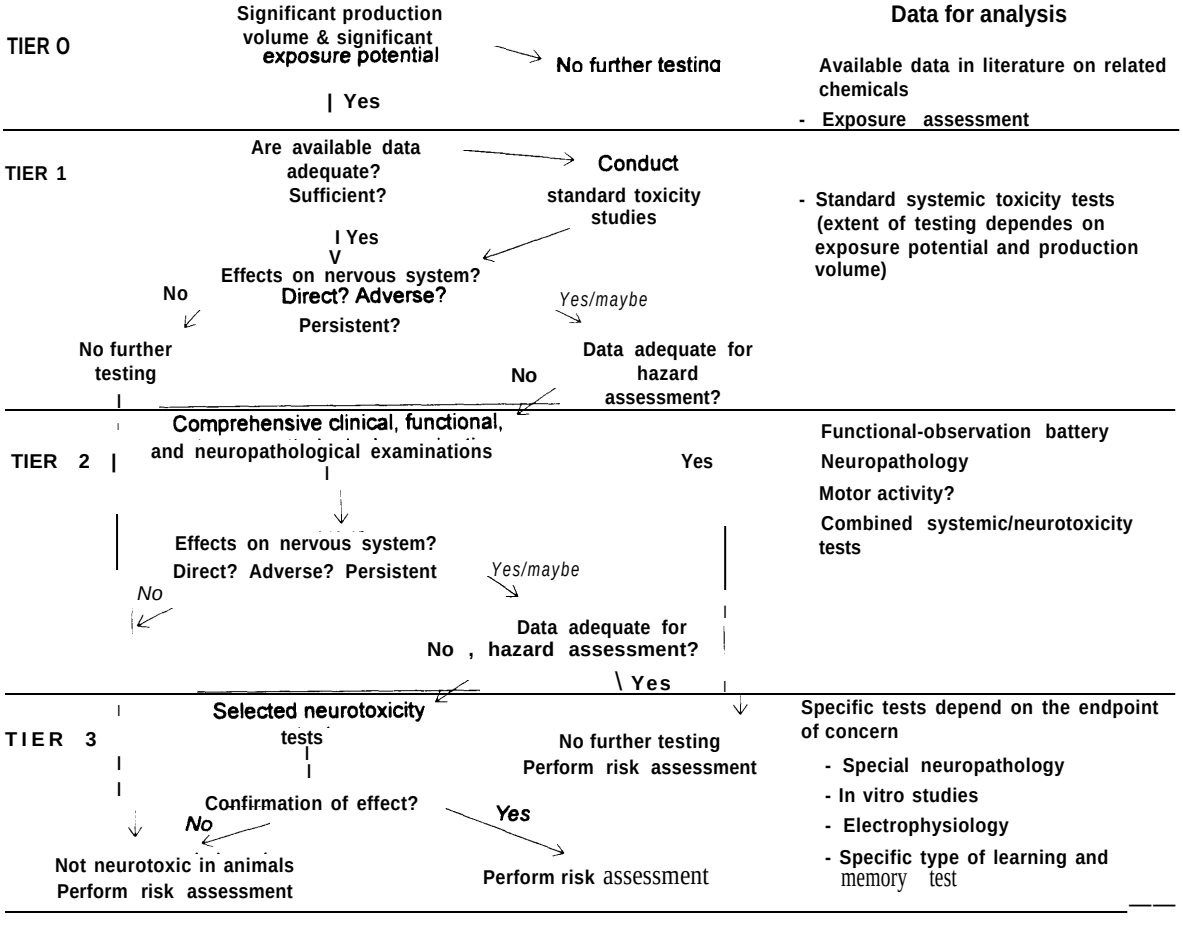
specific test results than those of the first tier and designed to characterize the nature of a chemical's neurotoxicity. The choice of the most appropriate approach and method(s) is dependent on the scientific questions generated by the results of first-tier testing. Such second-tier tests are aimed at objectively quantifying sensorimotor deficits, evaluating cognitive behaviors relating to learning and memory, and assessing performance of complex tasks.

Third-tier testing involves "mechanistic studies" which attempt to establish a detailed profile of a chemical's effect at several levels of nervous system organization (i.e., behavioral, cellular, molecular). Such tests are expected to provide data on enzyme function, ionic balance, transmitter systems, receptor modulation, and the pathogenesis of effects. The value of mechanistic studies cannot be emphasized enough. It is from such studies that understanding of the processes underlying neurotoxicity and specificity of effect is gained. Mechanism of action studies provide the basis for moving beyond empirical structure-activity analysis and being able to rationally prioritize chemicals for testing and most importantly, develop biologically-based models of neurotoxicity.

The EPA has established guidelines to test existing chemicals under TSCA (16). These guidelines include a FOB, motor activity, neuropathology, nerve conduction velocity, and schedule-controlled operant behavior. A neurotoxicity screening battery (17) combining the FOB, motor activity, and neuropathology guidelines into a single screening battery is now required for registration and reregistration of pesticides. However, the EPA does not at present use a tier-testing strategy within the TSCA regulatory context. For example, current test rules are promulgated with a full battery of tests with no guidance on how to use tests in a tiered manner. Likewise when testing is completed for a chemical, all test results from hazard identification, characterization, and mechanism-based studies are considered together (figure 4-1) to determine a critical adverse effect (the most sensitive endpoint). The critical effect could be identified from any of the data available (including the FOB) and the risk assessment process then uses this effect to support regulatory decision making.

Within the regulated and basic science communities, the concept of a tiered approach to testing has received wide support. A scheme for

FIGURE 4-2: Test Strategy for Neurotoxicity



SOURCE: Adapted from Eisenbrandt, D. L., et al., "Evaluation of the Neurotoxic Potential of Chemicals in Animals," *Food and Chemical Toxicology* 32:655-669, 1994.

using data collected by tiered testing (figure 4-2) which begins with the collection and analysis of data from standard toxicology tests has been published (4); Perhaps the strongest disagreements that the regulated community have with the present regulatory approach to neurotoxicity testing are that the data from standard tests are underutilized as a first-tier test for neurotoxicity and that relatively nonspecific behavioral signs from existing data have been used to trigger additional testing (which is often nonspecific as well) and risk assessments. The EPA, on the other hand, is concerned that some first-tier approaches involving cageside observations may be insensitive and therefore, subject to frequent false negative results. An additional concern is that cageside observations collected during standard toxicity tests have not been designed to specifically detect neurotoxicity. Incorporation of more systematic, better defined protocols for cageside observations into standard tests may provide a wealth of first-tier type information.

The significant costs associated with current screening methods recommended for existing chemicals under TSCA (FOB, motor activity, and neuropathology) are an obstacle to widespread

use of the methods. For example, addition of the three screening tests to an acute oral toxicity has been estimated to increase median cost of the base test ($21K) by $50K (15). For subchronic tests, the base test cost ($111K) has been estimated to increase by $79K; the base chronic test cost ($308K) has been estimated to be increased by $113K (15). The addition of schedule-controlled operant behavior to a subchronic oral test has been estimated to increase test cost by $64K (15). When neurotoxicity testing is conducted as a independent test, final costs have to include both those associated with the neurotoxicity test and the incremental original base cost. In the future, the use of *in vitro* methods could significantly reduce the costs of first-tier testing.

DESIGN CRITERIA FOR NEW SCREENING TESTS

A realistic assessment of how many chemicals actually might require testing is also important in designing future tests. If all of the approximately 72,000 chemicals on the TSCA inventory were to be tested by the current screening battery (FOB, motor activity, and neuropathology), only for acute effects, the testing bill would be greater than $4B; not to mention the 6 billion animals that would be needed. This is clearly not a feasible approach. However if the number of chemicals were actually more manageable, more significant testing might be achieved. The calculation of a realistic number of chemicals for testing could be made by subtracting the 25,000 polymers in the inventory and the large number of site-limited intermediate chemicals, low production volume chemicals, those with little or no exposure, and those which cannot be tested because of physical-chemical property limitations. With a realistic evaluation of the number of chemicals requiring testing, the magnitude of the problem may be much more manageable than it currently is perceived to be. Clearly, some strategy for prioritization of chemicals for testing needs to be developed. The prioritization process could begin with the EPA list of chemicals reported under the TSCA 8(b) Inventory Update Rule, which includes 9487 chemicals produced in excess often-thousand kilograms/year (1) and set priorities for screening a smaller set of chemicals based on exposure potential.

Screening tests for neurotoxicity should have several characteristics common to testing paradigms for other forms of systemic toxicity. The methods should have a high degree of sensitivity to insure against excessive numbers of false negative results. The method should be specific and produce results which are predictive of a hazard to the nervous system and thus avoid frequent false positive results. The results should be reproducible within and between laboratories. The screen should be cost effective and produce timely results; assays which cost thousands of dollars and take months or years to yield results are not really screening tests. If the screening method is to be widely used, most toxicology laboratories should be capable of performing the method with trained personnel.

Any attempt to design additional screening methods needs to take into consideration that many of the materials tested will not have neurotoxic potential. It is therefore critical that the methodology used be fairly specific for neurotoxicity to prevent a high number of false positive results. Any misclassification of chemicals for neurotoxicity, whether false positive or false negative, will result in some form of unnecessary future cost and wasted resource. The available estimates of the number of chemicals which might be neurotoxic ranges from 5-28°/0 (15). A significant reason for this range of estimates in the number of neurotoxic substances is in how neurotoxicity, and in particular, an adverse effect on the nervous system is defined. Thus the designer of future screening tests will need to decide whether the test is meant to detect any perturbation in baseline function of the nervous system or generally recognized toxic effects on the nervous system.

If a screening test is expected to be used for new chemicals as well as existing chemicals, the test designer should consider where in the product development cycle the chemical is to be tested. The cost associated with current neurotoxicity test

methods are such that the tests are only used for chemicals which are produced in relatively large volumes. However, there are potential uses for methods to screen for neurotoxicity during the product development cycle. Most product development cycles can be broken down into various phases such as product conception, product and process development, commercialization, and post-commercialization. Ideally, screening tests could be used early in product conception and development phases of the cycle as an aid to choosing candidate chemicals for development. Since *in vitro* assays require less test material, they may be particularly useful in early phases of product development when supplies of new chemicals are typically low. Use of such screening tests in earlier phases of product development could support pollution prevention evaluations along with accompanying product efficacy, cost benefit, process development, and other important considerations.

ALTERNATIVES TO EXISTING TEST METHODS

All presently available neurotoxicity test guidelines for existing chemicals use laboratory animals, primarily rats. In order to accomplish screening of large numbers of chemicals, alternative methods need to be developed to reduce the cost and time to complete screening. Such methods include structure-activity analysis as well as *in vitro* methods.

In general, structure-activity relationships (SAR) in neurotoxicology have received relatively little attention, however, SAR is routinely used in the premanufacture notification process for new chemicals and by pesticide and pharmaceutical research groups for the identification of candidate chemicals with neuroactive properties. There are some examples of SAR being used effectively in neurotoxicology. Many of these are based on available mechanistic data for representative chemicals that allow for an understanding of a specific process underlying one type of neurotoxicity which can be generalized to other similar chemicals. For example, the identification of the importance of gamma-diketones for induction of axonopathy led to the screening of chemicals which were gamma-diketones or could be metabolized to gamma diketones for axonopathy using small scale animal screening tests (8) and *in vitro* techniques (14). SAR techniques are currently used empirically to qualitatively identify materials which might be neurotoxic. As currently used, SAR is not able to identify chemicals which are not neurotoxic. However, there is reason to believe that continued work on SAR could lead to much more informative quantitative techniques. SAR offers the potential for development of inexpensive technology that could be used to evaluate large numbers of chemicals before other screening tests are employed.

For development of improved SAR techniques and *in vitro* methods, there is a need to better understand the chemical-biological interactions (mechanisms) that result in neurotoxicity. If *in vitro* tests are mechanistically-based, they are much more likely to be used earlier in the product development process as they will more likely be accepted as reliable predictors of neurotoxicity. Because of the number of chemicals to be evaluated and the complexity of the nervous system, mechanistic *in vitro* studies can be expected to provide results which can be interpreted and extrapolated. Due to the complexity of the nervous system, batteries of *in vitro* tests will be necessary to characterize toxicity and evaluate potential hazard. Even when batteries of *in vitro* tests are available for hazard identification, whole animal tests will probably still be needed to develop data sets adequate for risk assessment. However, the additional information provided by *in vitro* tests may reduce the number of animal required for first-tier testing.

As more is learned about the mechanism of action of neurotoxic chemicals, initial efforts should be directed at refining existing test methods to reduce the number of animals used to evaluate neurotoxicity. Such information would also offer the ability to develop *in vitro* assays that would address specific mechanistic endpoints

Table 4-1: In Vitro Assays for Neurotoxicity

Test System	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Example
Membrane models (erythrocyte and synaptosome membranes)	Effects on integral cell membrance enzymes (AcHE, ATPase)	(+) Useful for mechanistic studies (-) Limited specifically to compounds which effect cell membranes	Carbon disulphide Toluene
Primary neuronal cultures		(+) Possible to study individual neurons (+) Useful for mechanistic studies (-) Neurons are deprived of their normal afferent and efferent targets (-) Maintenance of the cells is difficult (-) No blood-brain barrier	Excitotoxic amino acids NMDA antagonists
Glial cell cultures		(+) Useful for mechanistic studies (-) No blood-brain barrier	Ethanol Alpha-chlorhydrin
Cell lines	Effects on ion channels and interaction with receptors	(+) Useful for studying cell biology (-) Model system that shares only certain features with real neurons or glia	Methylmercury Pyrethroid insecticides
Organotypic explants	Effects on development of the nerve system, on development of neuro-muscular junctions or other morphological endpoints	(+) Useful for mechanistic studies (-) Preparation and maintenance is difficult (-) Neurons are immature (-) Explant is disconnected from its normal afferents	Tellurium Hexacarbon solvents
Rotation-mediated aggregating cultures	Effects on specific transmitter systems, on cell surface recognition and on enzymes	(+) Ease of preparation, reproducibility and representation (+) Appropriate for interdisciplinary investigation (-) Neurons are immature (-) Large quantities of foetuses are required (-) Electrophysiological examination is not possible	Kainic acid 6-hydroxydopamine

SOURCE: Adapted from European Center for Ecotoxicology and Toxicology of Chemicals, Monograph No. 18, *Evaluation of the Neurotoxic Potential of Chemicals* (Brussels: September 1992)

responsible for neurotoxicity. In cell culture systems, it is possible to examine the effects of growth factors, hormones, and chemicals on growth, differentiation, cell-cell interactions, and metabolic activities. In recent years, the advent of molecular biological methods has allowed for cell lines to be developed to examine specific targets as neurotransmitter receptors or specific genes. Because the nervous system is composed of a highly specialized, heterogeneous, yet integrated population of cells, single *in vitro* test systems are unlikely to be able to mimic the responses of the nervous system to a broad range of chemically-induced toxicities. However batteries of *in vitro* tests offer the possibility of developing first-tier screening methods.

Within the area of neurotoxicology, recent evaluations have focused on correlating *in vivo* and *in vitro* endpoints. Although cell culture models have been proposed as systems for neurotoxicity screening, it is the ability to conduct detailed analysis and experimental manipulations that makes such culture systems attractive for the identification and subsequent evaluation of cellular mechanisms underlying neurotoxicity. The major types of nervous systems cultures (table 4-1) that have been useful in assessing neurotoxicity range from clonal cell lines, primary cells, reaggregate cultures, organotypic explants, organ cultures to whole embryos. Each system offers a unique approach to examining toxicant-induced perturbations, however, each system is not without distinct limitations. The emphasis on the use of *in vitro* techniques within neurotoxicology has resulted in the development of model systems which encompass a wide array of basic approaches both as a screening battery for early detection of potential neurotoxicity and to detect basic underlying mechanisms associated with both neural development and functioning. While *in vitro* systems offer unique opportunities to examine detailed cellular events associated with environmental perturbations to the nervous system, the results from such studies need to be viewed in the isolated nature in which they are generated. If a chemical is found, *in vitro,* to have selective neurotoxic properties as compared to general cytotoxicity one may speculate that the chemical would also be neurotoxic *in vivo.* However, no matter how attractive and useful an *in vitro* system appears to be, it is still an artificial system that is isolated from the various biological processes that greatly modulate *in vivo* neurotoxicity. Results from *in vitro* studies using single cell systems are not easily extrapolated to an integrated nervous system. In addition, the interpretation of *in vitro* data collected in the absence of normal metabolic systems and without appropriate toxicokinetic and toxicodynamic information is highly problematic. Given the complicated nature of the interdependent interactions of the various cell types and network processes in the nervous system, it would be unwise to at this time to conclude that a chemical has or does not have neurotoxic potential based upon data from *in vitro* systems alone.

VALIDATION OF NEW SCREENING METHODS

Numerous test methods exist to evaluate the potential for a chemical to produce neurotoxic effects by alteration of specific organization processes in the nervous system (2, 11). The question of validation of these systems remains a difficult problem. For example, many laboratories have ongoing projects to develop methods for screening chemicals, however, assays that have been found to be useful and predictive in one laboratory for a specific purpose and in an isolated environment may not be considered "validated" for broad screening purposes by other laboratories. Such assays can include both *in vivo* behavioral screening assays and mechanistically-based *in vitro* tests. The success of such tests is critically dependent upon the level of expertise and training that exist within any one laboratory. In order to validate an assay for widespread use, there are a number of steps that are required. Among these are that the assay must have adequate development to be considered robust enough to be used under varying laboratory conditions without failure and the assay must receive acceptance following a peer review which

typically includes a round of interlaboratory comparison testing.

There are many approaches to the assessment (validation) of methods. A modular approach has recently been submitted for publication (6,7). The concept prescribes validating a single *in vitro* assay independent of other *in vitro* assays. This modular concept evaluates the results obtained with a specific group or class of chemicals in an *in vivo* assay (validation standard). The same group of chemicals are evaluated for their response in an *in vitro* assay. The results of the *in vivo* and *in vitro* **assays** are compared to assess whether the *in vitro* assays predict the *in vivo* response. A module consists of the chemical group, the validation standard, and an *in vitro* assay. A validation study may consist of several modules. In this case, one evaluates each module separately and therefore, an *in vitro* assay is not compared to another *in vitro* assay. Validation is an important step in the development of acceptance of alternative methods for testing. Without broad acceptance by the neurotoxicology testing community, new screening methods are unlikely to receive widespread use (6,7).

CHAPTER 2 REFERENCES

1. Auer, C., Chemical use inventory update meeting for stakeholders, Washington, DC, April 13, 1995.
2. Chang, L. W., and Slikker, Jr., W. (eds.), *Neurotoxicology: Approaches and Methods* (Orlando, FL: Academic Press, Inc, 1995).
3. Consumer Product Safety Commission, "Labelling Requirements for Art Materials Presenting Chronic Hazards; Guidelines for Determining Chronic Toxicity of Products Subject to the FHSA; Supplemental Definition of Toxic under the Federal Hazardous Substances Act," final rule, *Federal Register* 57:46626-46674, *1992.*
4. Eisenbrandt, D. L., et al., "Evaluation of the Neurotoxic Potential of Chemicals in Animals," *Food and Chemical Toxicology* 32:655-669, *1994.*
5. European Centre for Ecotoxicology and Toxicology of Chemicals, *Evaluation of the Neurotoxic Potential of Chemicals,* Monograph No. 18 (Brussels, Belgium: September 1992).
6. Goldberg, A. M., et al., "Framework for Validation and Implementation of *in vitro* Toxicity Tests," *Journal of the American College of Toxicology* 12:23-30, *1993.*
7. Goldberg, A.M., Epstein, L.D., and Zurlo, J., "A Modular Approach to Validation," *In Vitro Toxicology,* forthcoming, 1995.
8. Krasavage, W., et al., "The Relative Neurotoxicity of Methyl-n-butyl ketone, n-hexane, and Their Metabolizes," *Toxicology and Applied Pharmacology* 52:433-441, *1980.*
9. National Research Council, Commission on Life Sciences, Committee on Neurotoxicology and Models for Assessing Risk, *Environmental Neurotoxicology* (Washington, DC: National Academy Press, 1992).
10. Nordic Council of Ministers, *Criteria Document for Evaluation of Existing Data: Occupational Neurotoxicity* (Denmark: 1992)
11. Shahar, A., and Goldberg, A.M. (eds.), "Model Systems in Neurotoxicology: Alternative Approaches to Animal Testing," *Progress in Clinical and Biological Research (New* York, NY: Alan R. Liss, Inc., 1987).
12. Tilson, H., "Neurotoxicology in the 1990' s," *Neurotoxicology and Teratology* 12:293-300, *1990.*
13. Tilson, H. A., MacPhail, R. C., and Crofton, K. M., "Defining Neurotoxicity in a Decision-making Context, " *Neurotoxicology 16;363-376, 1995.*
14. Tsukamoto, T., and Yonezawa, T., "Neurotoxic Effect of 2,5-hexanediol *in vitro,"* *Clinical Neurology* 18:697, 1978.
15. U.S. Congress, Office of Technology Assessment, *Neurotoxicity: Identifying and Controlling Poisons of the Nervous System,* OTA-BA-436 (Washington, DC: U.S. Government Printing Office, April 1990).

16. U.S. Environmental Protection Agency, "Neurotoxicity Functional Observational Battery; Motor Activity; Neuropathology; Schedule-controlled Operant Behavior," *Federal Register* 50:39458-39466, *1985.*
17. U.S. Environmental Protection Agency, *Pesticide Assessment Guidelines, Subdivision F: Neurotoxicity* (Washington, DC: U.S. Government Printing Office, March 1991).
18. U.S. Environmental Protection Agency, Interagency Committee on Neurotoxicology, "Principles of Neurotoxicology Risk Assessment," final report, *Federal Register* 59:42360-42404, *1994.*