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GUIDANCE DOCUMENT FOR NEUROTOXICITY TESTING

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GUIDANCE DOCUMENT FOR NEUROTOXICITY TESTING

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INTRODUCTION

5.   This document is intended to provide guidance on strategies and methods for testing of chemicals for potential neurotoxicity. The primary objective of this guidance is to ensure that necessary and sufficient data are obtained to enable adequate evaluation of the risk of neurotoxicity arising from exposure to a chemical. This document does not specifically address developmental neurotoxicity testing as this area will be covered by another Guidance Document (Draft OECD Guidance Document on Reproductive Toxicity Testing and Assesment) and a Test Guideline (Draft OECD 426).

6.   An iterative assessment/testing strategy is recommended. To minimise animal usage and optimise allocation of resources, data should be assessed *before* each iteration of testing to decide if they are adequate to evaluate the risk arising from the intended use of the chemical, or if further testing is needed. Because assessment of data is an essential part of the overall strategy, this document provides a definition of neurotoxicity, and discusses different types of neurotoxic effects. Guidance is provided on test methods, because familiarity with these methods is required to assess data or to select methods to identify and/or characterise neurotoxic effects.

7.   The document constitutes an essential supplement to those existing OECD Test Guidelines (Appendix 1) that can be used to obtain information on the potential neurotoxicity of chemicals. Specific OECD Test Guidelines include those for single dose toxicity (e.g. OECD 402, 403, 420, 423 and 425), repeated dose toxicity (e.g. OECD 407 and 408), as well as Test Guidelines specifically developed for the study of neurotoxicity in adult and young laboratory animals *[OECD Test Guideline for Neurotoxicity (424), and OECD Test Guidelines (418 and 419) for Delayed Neurotoxicity of Organophosphorus Substances and OECD Test Guideline for Developmental Neurotoxicity (426), OECD Test Guideline for Combined Repeated Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Test(422)]*. Studies conducted under other OECD Guidelines for systemic toxicity testing could also provide relevant information.

8.   A number of organizations in several nations have written documents on the evaluation of neurotoxicity data.   An ECETOC monograph (1992) served as the basis of the first draft of this guidance document. It included a discussion of definitions, issues of transient and indirect effects, an extensive annotated review of test methods, and a testing strategy. The Nordic Council of Ministers (1992) issued a report arising out of a working group that sought to define criteria for identifying and classifying neurotoxic chemicals. Criteria are provided that define a process for assigning chemicals to groups depending on the available evidence, e.g., probably neurotoxic, and description of the potency of chemicals. It was meant to serve as a tool for generating lists of hazardous chemicals. The Danish Environmental Protection Agency (1995) issued a particularly thorough document including definitions, methodology, and criteria for evaluation of neurotoxicity. Two more recent U.S. government documents (U.S. EPA, 1994, 1998) were written as principles of and guidelines for neurotoxicity risk assessment. They also discuss general definitions and issues, an overview of test methods, and the interpretation of data within the U.S. framework for risk assessment, i.e. hazard assessment, dose response assessment, exposure assessment, and risk characterization.

PRINCIPLES OF NEUROTOXICITY HAZARD ASSESSMENT

9.   A definition of neurotoxicity (or a neurotoxic effect) is an adverse change in the structure or function of the nervous system that results from exposure to a chemical, biological or physical agent. This definition hinges on interpretation of the word 'adverse' (OTA, 1990). Disagreement exists among scientists as to what constitutes an 'adverse change'. A practical definition of an 'adverse change' is: "any treatment-related alteration from baseline that diminishes an organism's ability to survive, reproduce or adapt to the environment" (US EPA, 1994). In particular, the term 'adverse' should be considered in a toxicological sense and implies a detrimental change in structure and/or interference with normal function of the nervous system.

10.   Adverse changes in structure or function of the nervous system may result from single or repeated doses of a chemical. Since both single and repeated exposures are possible scenarios for human exposure, the neurotoxicity assessment/testing strategy must consider both situations.

11.   Adverse changes in the nervous system can be direct, due to an agent acting directly on target sites in the nervous system, or indirect, by acting on target sites outside the nervous system which subsequently affects the nervous system. Such indirect changes may be considered adverse, but not necessarily neurotoxic (US EPA, 1994; Robins & Cotran, 1979). The indirect effect of substances on the nervous system is of special concern in single-dose animal studies performed at very high dose levels. At near lethal doses, tremor, ataxia and convulsions are often observed, although the primary target is not the nervous system. Other indirect causes include insufficient oxygen or nutrient supply to the brain and accumulation of toxicants in the blood and brain because of failure in kidney and liver function. However, it may be very difficult to differentiate between direct and indirect effects based on the results of only a single study.

12.   The assessment of neurotoxicity should incorporate a level of concern based on the type, severity, number and either full or partial reversibility of the effect(s). For example, convulsions generally cause a higher level of concern than drowsiness, and paralysis generally causes a higher level of concern than does mild weakness. Likewise, a pattern or cluster of related effects generally causes a higher level of concern than individual or unrelated effects. Chemicals which produce a clear and consistent pattern of neurotoxicity at lower dose levels than other organ/system toxicity are generally of higher concern than chemicals which produce only a few unrelated effects. However, the observation of some specific endpoints, even of limited duration in time (e.g., body tremors, convulsions) may be sufficiently important to raise a high level of concern, even if they are the only observable change.

13.   Reversibility of effect is a particularly important factor in the level of concern associated with a neurotoxic effect. Neurotoxic effects may be irreversible i.e. cannot return to the state prior to exposure, resulting in a permanent change in the organism, or reversible i.e. can return to the pre-exposure condition, allowing the organism to return to its normal state prior to exposure. Clear or demonstrable irreversible changes in either the structure or function of the nervous system cause greater concern than do reversible changes.

14.   If neurotoxic effects are observed at some time during the life span of the organism but are slowly reversible, the concern is also high. In general, there is lesser concern for effects that are rapidly reversible or transient (i.e. measured in minutes, hours, or days, and appear to be associated with the pharmacokinetics of the causal agent and its presence in the body). However, the nervous system has reserve capacity and the reversibility of effects does not exclude the possibility that cell death has occurred.

In addition, the context of the exposure should be considered in developing a level of concern for reversible effects. For example, there may be higher concern for reversible changes that occur in the occupational setting or environment, where even brief sedation could interfere with operation of heavy equipment in an industrial plant. Thus, even reversible neurotoxic changes should be evaluated carefully to establish an appropriate level of concern.

OVERVIEW OF THE ASSESSMENT/TESTING STRATEGY

15.   An iterative assessment/testing strategy is recommended, in which all available information is assessed before conducting the next iteration of testing. The testing strategy should allow for flexibility, so that test programs can be developed on a substance-by-substance basis according to relevant information (e.g., physicochemical properties, structure activity relationships, toxicology data, and information from recorded human exposure, the proposed use of the individual chemical, and the likely route of human exposure). The iterations also include periodic review of data from animal studies and human exposures which appear after the test program has been completed.

16.   Toxicity data from structurally-related chemicals can be an important source of information, especially for the initial assessment. This information may come from the presence or absence of changes in behavioural, biochemical, physiological, and morphological endpoints. The initial assessment may indicate a need for additional information, which is obtained from systemic toxicity tests. The nervous system supports a plethora of different functions in the body, and it is not usually possible or practical to obtain in-depth information about all possible endpoints in a single study. Instead, the initial test(s) most often include so-called "screening studies", which are conducted at high doses with simple endpoints to delineate system/organ-specific effects. However, if available data provide a significant clue as to possible neurotoxic effects, then additional endpoints may be included in the initial test(s) to provide in-depth information about a specific type of neurotoxic effect.

17.   If the available information [historical data and the initial test(s)] do not give any evidence for potential neurotoxicity, then the initial test(s) may be sufficient to complete a hazard assessment for neurotoxicity. In other cases, assessment of the information available after the initial test(s) indicate the need for iterations of testing and assessment. In designing these subsequent tests, it is important to minimise the number of simple endpoints which merely duplicate those obtained during the first iteration of testing. Instead, these tests should include specific, in-depth endpoints needed to characterise neurotoxic effects, to provide perspective for equivocal functional or morphological signs of neurotoxicity, or to clearly differentiate neurotoxic effects as direct neurotoxicity or secondary to other toxicities (e.g., organ-specific toxicity).

Initial Testing

18.   The first animal data for neurotoxicity assessment are most often provided by standard single dose (OECD 402, 403, 420, 423 and 425) or repeated dose toxicity studies (OECD 407, 408) where functional and/or histopathological information is gathered on all major organ systems, including the nervous system.

19.   When available information provides indications of possible neurotoxic effects, additional endpoints may be included in the initial standard tests(s) (see paragraph 11) in order to obtain in-depth

information about a specific type of neurotoxic effect. Alternatively, existing information may indicate a need to conduct a neurotoxicity study (OECD 424) with specific tests (see Appendix 2) to assess a suspected neurotoxic effect. The decision to add specific endpoints to the initial study should take into consideration the potential for confounding toxicological effects of the higher doses usually employed in initial studies.

20.   In many cases, the initial repeated dose study will be conducted after the first single-dose study. In this situation, it may be possible for the results of the repeated dosing study to provide data relevant for assessing the risk of a single dose. In general, these data would be provided by including observations of behaviour or other endpoints immediately after dosing. However, one limitation of repeated dose studies is the potential to develop tolerance, sensitization or other compensatory mechanisms. Since standard protocols for repeated dosing studies do not call for evaluation of acute effects, acute toxicity may be underestimated unless the modifications of the repeated dosing protocol include observations after the very first dose.

Assessment of Initial Test Data

21.   Single-dose toxicity tests can provide important data for evaluating the risk arising from the intended use of the chemical. However, one limitation of initial single dose tests is that they are typically conducted at lethal or near-lethal doses, which may make it difficult to determine whether observed functional effects are direct or indirect (see paragraphs 7-9).

22.   Initial repeated-dose studies are generally conducted at doses lower than those used in the initial single-dose studies. In general, signs of toxicity to the nervous system and other organ systems evolve more slowly at these lower doses, so that there is a better opportunity to compare their evolution, and thus differentiate direct from indirect (see paragraphs 7-9) effects on the nervous system.

General Principles

23.   The need for iterations of testing should be considered on a case-by-case basis using all the available information. These follow-up studies should be conducted only after it has been determined that the existing data are not adequate for evaluating the risk based on the intended use and foreseeable misuse of the chemical. Factors to consider when assessing the need for additional testing include:

> (a) **The level of concern.** Chemicals which generate a high level of concern for neurotoxicity generally require more iterations of testing than those with lower levels of concern. When there is no evidence of effects on the nervous system from systemic toxicity studies in laboratory animals or from human exposure or from SAR, then the general level of concern is low.
>
> (b) **A need to differentiate neurotoxic effects as direct or indirect.** Careful consideration should be given to the need for additional anatomical and clinical pathological endpoints for other organ systems that might help to differentiate direct from indirect effects.
>
> (c) **A need to provide perspective for equivocal morphological or functional signs of neurotoxicity.** Careful consideration should be given to the need for additional doses, additional subjects, or more refined functional or morphological procedures (e.g., perfusion fixation, histochemical procedures, morphometry).
>
> (d) **A need to characterise neurotoxic effects.** If there is clear evidence of neurotoxicity, and data are sufficient to enable the evaluation of risk arising from the intended use of the chemical, then normally no further testing is required. Where more data are needed for risk assessment purposes, usually as a first step, characterisation of the type of neurotoxicity is warranted (NCM-NIOH, 1992; Simonsen et al., 1994; ECETOC, 1992; Eisenbrandt et al., 1994). Characterisation of effects could include specialized behavioural, morphological,

neurochemical or neurophysiological measures (see Appendix 2). This characterisation may be necessary to define the most sensitive parameters which then could be used to establish sound data for risk assessment. Using these defined parameters, NOAELs or LOAELs can be determined for different exposure-driven scenarios, e.g, in experiments with a single exposure or in studies with longer duration or for various routes of exposure (oral, dermal, inhalation) or for special exposed populations (e.g., based on gender, age or animal strain). The tests used for characterisation (see Methods section below) need to address specific aspects of neurotoxicity that are related to the structure and function of the nervous system and should contribute to the differentiation between adverse effects and other treatment-related effects if applicable.

(e) **The shape of the dose-effect curve and the need to establish a no-observed-adverse-effect-level for neurotoxicity.** If the dose-effect curve is steep (e.g., high dose produces clear signs of neurotoxicity, but two lower doses produce no signs), then there may be less need to conduct an additional iteration of testing. If the dose-effect curve is shallow, then there may be a greater need for additional iterations of testing to define a NOAEL.

(f) **The routes and likelihood of human exposure resulting from the intended use or foreseeable misuse of the chemical.** Decisions about additional iterations of testing should include a careful consideration of the size of the population exposed to a chemical, as well as the likelihood that the exposed population will actually absorb the chemical. Some chemicals (e.g., pharmaceuticals or crop pesticides) have the potential for significant intentional or unintentional human exposure, while others (e.g., site-limited chemical intermediates, materials incorporated into articles with limited potential for release) provide very limited or no potential for exposure. Different chemicals also have vastly different absorption from different routes of exposure. For example, some materials have little likelihood for absorption, while others may be readily absorbed by multiple routes. The decision to conduct an additional study, as well as the dose route and duration of the study, should consider these factors carefully.

(g) **Special considerations of the exposed population.** When designing additional iterations of testing, careful consideration should be given to any special characteristics (e.g., age, gender, strain) of the exposed population.

24.   In designing these follow-up studies, careful consideration should be given to dose levels and study conditions that minimise confounding effects of generalised systemic toxicity. Careful consideration should be given to minimise the number of endpoints which simply replicate information available from previous studies.

Experimental Design and Selection of Methods

25.   Neurotoxicity data can be acquired using a vast array of behavioural, neurological, neurochemical and morphological techniques (see Appendix 2). This fact underscores the importance of using the

professional judgement of the competent authority and other interested parties together with the contractor/laboratory to determine the design of tests.

26.   Selection of the most appropriate tests should be determined on a case-by-case basis and be guided by all of the available information of the chemical. Some tests might be affected either directly or indirectly by changes in non-neural organs as well as by other factors (e.g., age, gender, rearing condition, hormone status, dietary restriction on either food or water), and the potential effects of such variables should be considered in the experimental design.

27.   In the design of the study (including the selection of test methods), the following points should be among those considered:

(a) Can all significant data needs be identified and listed? Does the study design address all of these data needs? If not, is more than one study required to address data needs?

(b) Are satellite groups needed to provide adequate numbers of subjects to measure all endpoints?

(c) Is the toxicity of the chemical well-characterised (e.g., do we know the part(s) of the nervous system affected by the chemical)? If so, can this information be used to focus the endpoints used to evaluate neurotoxicity?

(d) Is significant toxicity to non-neural organs expected? Does the study design attempt to provide a sufficient dose range and other data to differentiate between systemic and neurotoxic effects?

(e) Does the study design take into account the effects of known variables (e.g., dietary restrictions, gender, rearing conditions (eg. group vs individual housing, sexual segregation vs mixed-sex rearing), ageing of subjects) that might influence the data?

(f) Is there value in using adjunctive tests (e.g., thyroid function, clinical chemistry) to help distinguish direct effects from indirect effects on the nervous system?

(g) Do negative results indicate the absence of adverse effect? Are there any individual endpoints for which a change would be considered as adverse?

(h) If reversibility is an issue, is the study design appropriate to evaluate reversibility or partial reversibility? Does the study design allow discrimination between transient and persistent effects?

(i) Can the results of this study/these methods be extrapolated to the human exposure condition? If not, should a non-rodent subject be considered? Should the route of exposure be changed?

(j) Are the test methods sensitive and specific? Is a quantitative result needed? Do the proposed methods permit an assessment of severity of effects?

28.   **Statistical Analysis:** Test results should be analyzed using generally accepted statistical techniques that are appropriate for the type of data (eg., parametric tests for continuous data, non-parametric tests for frequency or rank data) and consistent with the experimental design, including repeated measures designs. The choice of analyses should consider the distribution of the measured variables well as the need for adjustments for multiple comparisons. Though there may be a number of techniques available, generally accepted statistical techniques should be employed and should be selected as part of the study design.

APPENDIX 1: OECD TEST GUIDELINES

29.   *This appendix identifies OECD Test Guidelines mentioned in paragraph 3: single dose toxicity (402, 403, 420 and 423), repeated dose toxicity (407, 408 and 422) as well as OECD Test Guidelines specifically developed for the study of neurotoxicity in young and adult laboratory animals (418, 419, 424 and 426). Studies conducted under other OECD Guidelines for systemic toxicity testing could also provide relevant information.*

30.   OECD Test Guideline 424 (Neurotoxicity Study in Rodents) includes (i) detailed clinical observations in the home cage and open field, (ii) functional tests including motor activity, and (iii) neuropathology using perfusion-fixed tissues. It uses basically the same tests (functional tests, clinical observations) as those conducted in OECD Guidelines 407 and 408 but employs a larger sample size than OECD Guideline 407, call for more frequent measurement of functional tests, requires that observations are conducted without knowledge of treatment level, and allows for a longer exposure period to be used. Unlike OECD Guidelines 407 and 408, OECD Guideline 426 requires perfusion fixed tissues. The OECD Neurotoxicity Guideline 424 recognises the possible redundancy in tests conducted in other studies and allows flexibility in designing neurotoxicity studies so that resource use can be optimised. It is important to minimise the number of simple endpoints which merely duplicate those already obtained from repeated dose toxicity studies. Instead, these tests should include specific, in-depth endpoints needed to characterise neurotoxic effects.

31.   OECD Test Guideline 424 was written assuming a repeated dose regimen, oral route of administration and that rat is the species of choice. It may be necessary to amend the guidelines for acute exposures, other routes of administration, particularly inhalation, and for other species (e.g. dogs, altricial and procial rodents. The clinical and functional test requirements in this guideline may require special considerations in order to conduct them under the time constraints imposed by acute toxicity testing (see, e.g., U.S. EPA, 1998a).

32.   Based on expected human use, there may be a need to conduct neurotoxicity testing in some studies using the dermal route, the inhalation route, or by other routes of exposure. These other routes of administration can impose scheduling or other constraints on the practical conduct of the study which may affect e.g. timing and frequency of observations. These need to be carefully considered in the design and conduct of the study.

33.   Dermal and inhalation exposures, which typically last at least 6 hours/day, 5 days/week, can make it practically difficult or impossible to conduct the detailed clinical observations and functional tests of all animals in a standard work day. A common practical solution for this type of study and for acute exposures is to divide the test subjects into a suitable number of replicates (e.g., 4-5), counterbalanced for exposure level and/or gender thus allowing for testing of more manageable and smaller groups.

34.   In addition, for repeated exposure studies, particularly inhalation studies where the complexity of the exposure system can impose a practical need to expose all subjects at the same time, it may be appropriate to consider testing on non-exposure day(s) (i.e. week-ends or additional planned non-exposure days).on   In such circumstances it may be necessary to extend the study so that the total number of exposures would be the total desired, e.g., 65 exposures for a subchronic study.

35.	Based on the study's focus, different approaches may need to be taken in establishing the time of testing in relation to the daily exposures during repeated exposure studies. For example, where cumulative toxicity is the primary focus, testing could precede the daily dose to rule out acute (less than 24 hour) effects. Alternatively, one may wish to focus on changes in acute effects across the duration of the study by testing at the same time after dosing. For dermal and inhalation studies testing is most practically performed after the daily exposure period although, exceptionally, it may be necessary to do some limited testing during the exposure period. Such requirements will significantly affect the design and schedule of the study.

APPENDIX 2: SPECIFIC TEST METHODS

NEUROBEHAVIOUR

What can be measured?

36.   Behavioural methods can be used to measure a wide variety of sensory, motor, cognitive, and autonomic functions (Tilson & Harry, 1992). This document catalogues methods that have been commonly used in neurotoxicological studies to assess these functions.

How functional changes are assessed

37.   Because most behaviours require the integrated activity of many components of the nervous system, many methods can provide information for more than one category of function. For example, pinching a rat's toe normally results in flexion of the leg and withdrawal of the foot. The presence of the flexor response provides information about sensing the pinch, whereas the strength of the flexor withdrawal provides information about muscle strength. Failure to flex in response to pinch could indicate either failure to sense the pinch, or inability to move the leg. Because interpreting results of any single behavioural tests may be problematic, behavioural methods are often effective when combined with additional behavioural methods or methods from other levels of nervous organization, i.e. electrophysiology, neurochemistry, neuroendocrinology, neuroimmunology, neuropathology.

General strengths of behavioural tests

38.   Behavioural tests are generally quantitative and non-invasive. Thus, the same animal can be tested repeatedly during a toxicity study to provide detailed information about the presence or absence of effects, severity of effects and the onset, duration or recovery of effects. Behaviour is the end-point of integrated systems, and even subtle alterations in any of the component systems are likely to be reflected in the modification of behaviour. Behavioural endpoints provide one of the most sensitive strategies to reveal subtle functional deficits. In addition, behavioural endpoints can uncover alterations in neural or extra-neural substrates for which no compensatory alternate behavioural response is available.

General limitations of behavioural tests

39.   Many changes in behaviour following exposure to chemicals may not be related to neurotoxicity. Within a single experiment, it may be difficult to quantify the contribution of neurotoxicity versus illness to observed effects. This may be particularly true in studies involving high doses. Due to the functional reserve capacity of the nervous system, there is the possibility of an animal suffering morphological damage but still functioning within normal limits (Mitchell & Tilson, 1982). It is also possible that a chemical could produce a behavioural change without a detectable change in the morphology of the nervous system. Moreover, not all behavioural changes necessarily represent the specific action of a chemical on the nervous system and some behavioural tests can be affected by changes in non-neural organs (Gerber & O´Shaughnessy, 1986; Rice, 1990) as well as by dietary restriction (Albee et al., 1987), hormonal state (Robbins, 1977), fatigue (Bogo et al., 1981), motivation (Cooper, 1981), developmental rearing conditions (Laviola, 1996; Laviola & Terrenova, 1998) or age (Soffie & Bronchart, 1988). Some behavioural tests may also require that the animals be exposed to external stimuli (e.g., electric shock)

which may be stressful, while others may require that the animals be deprived of food or water. Tests using natural or species–relevant stimuli (eg., stimuli emitted by predators, sexually receptive partners) and natural behaviours (e.g., ultrasonic vocalization in rats pups, maternal behaviour) may also be considered in evaluating neurotoxic effects (Alleva, 1993; Vitale & Alleva, 1999). These motivational considerations may complicate the design of the study. Some tests may also require special equipment and expertise of the study director.

GENERAL GUIDANCE

40.   In designing studies and interpreting effects, it is strongly recommended to consult with at least one scientist who has practical experience in conducting the specific behavioural test and interpreting the results from the procedure.

41.   Because many variables can affect behaviour on a single test, it is prudent to try to use different methods to obtain effects which can be correlated. This can be accomplished either by conducting additional behavioural tests which evaluate the same or similar endpoint(s), or by collecting additional measures of different types of endpoints (e.g., electrophysiological, neurochemistry, neuroendocrinology, neuroimmunology, neuropathology). For example, peripheral motor nerves can be assessed with forelimb and hindlimb grip strength, peripheral nerve conduction velocity, indirect evoked muscle potentials, needle electromyography and histopathology.

SPECIFIC GUIDANCE

Methods Commonly Used to Measure Motor Function (Table 1)

42.   Many tests of motor function are relatively cost-effective, are included in, or are compatible with standard toxicity studies, and are available in many laboratories. While little or no animal training is required, the ability or willingness of individual animals may vary, and this could lead to insensitivity or inappropriate interpretation of the data. The ability of the animals to perform on these tests can also be affected by non-neural factors, including: sickness, dietary restriction, and time of day (i.e., circardian rhythms). There have also been a number of tests developed to characterise chemical-induced neuromotor deficits, including swimming performance (Alder and Zbinden, 1983; Spyker et al., 1972), gait analysis (Jolicoeur et al., 1979; Schallert et al., 1978), inclined plane (Fehling et al., 1975; Graham et al., 1957), rope climbing (Carlini et al., 1967) and tremor analysis (Gerhart et al., 1985; Hudson et al., 1985; Tilson et al., 1985). Measures of vertical and/or horizontal movements, (e.g., motor activity) represents a broad class of behaviours involving coordinated participation of sensory, motor and integrative processes. Assessment of motor activity is non-invasive and has been used to evaluate the effects of acute and repeated exposure to many chemicals (MacPhail et al., 1989; Maurissen and Mattsson, 1989). Because motor activity changes with circadian rhythms, testing should be conducted in a specific phase of the animal's light/dark cycle and within a limited range of hours within the cycle.

Methods Commonly Used to Measure Sensory Function (Table 2)

43.   Sensory systems include those for vision, audition, taste, olfaction, thermoregulation, somatosensation (e.g., pressure, light touch, limb position) and nociception (painful stimuli). Simple tests such as the tail flick or hot plate are used routinely to measure chemical-induced changes in nociception. More complex operant behavioural procedures have been used to determine changes in thresholds to aversive stimuli or changes in somatosensory thresholds. Some chemicals produce ototoxicity, so that procedures such as reactivity to an acoustic stimulus have been developed to assess sensory function. More complex tests such as pre-pulse modification of the acoustic startle response and operant procedures to assess auditory thresholds have also been used. Tests of olfaction and taste are available, but have not been commonly used in routine neurotoxicological testing.

44.   Caution must be exercised in the interpretation of studies on sensory function because the endpoint that is measured is typically a motor response to a sensory stimulus. Motivation to respond to such stimuli is also affected by rearing conditions and/or previous stress experiences. Chemicals that affect motor function or the motivation to respond to such stimuli may confound measures of sensory function.

Methods Commonly Used to Measure Cognitive Function (Table 3)

45.   The term "cognition" implies awareness and includes many aspects of perception, thinking and remembering. Thus, there is no single test of "cognition". Instead, neurotoxicologists use tests which require components of cognition (e.g., learning and memory) to be used during the test. A wide variety of tests to assess chemical effects on cognitive functions (learning and memory) in animals is available (see Table 3). Measures of cognitive function may also be influenced by sensory, motor and motivational variables (Tilson et al., 1980; Pryor et al., 1983). The interpretation of data from studies on cognitive function should consider these variables by using the appropriate control procedures or conducting independent assessments (Eckerman et al., 1980). Less complex tests of cognitive function are not usually designed to control for or study independently such non-cognitive variables, while more complex tasks that require additional training of the animals or specialised equipment can often be designed to incorporate control procedures for non-cognitive variables.

46.   Learning and memory are theoretical concepts that are sometimes difficult to separate experimentally. Some tests are designed to emphasise one or the other. Learning is defined as the process by which new information is used to modify subsequent responding, while memory is the use of already acquired information to modify subsequent responding. One form of learning is habituation, which is a measure of a change in responding to a stimulus or a set of environmental stimuli usually during the same or temporally related test session(s). Some cognitive tests are capable of measuring learning as a function of a few trials during a training session such as the conditioned taste aversion and passive avoidance tasks, while others tasks such as active avoidance and some mazes require several training trials over days or weeks. Operant tasks such as discrimination learning, matching-to-sample (Paule et al., 1998) and repeated acquisition (Cohn and Paule, 1995) typically require several weeks of training. Sometimes, animals may receive several days or weeks of conditioning before the final procedure is implemented. Testing with tasks using single-trial training (eg., passive avoidance tasks) should be paralleled by tests with tasks that requiring more than one single training trial.

47.   Many of the commonly used tests of learning and memory require that animals be trained to make a response to receive positive or avoid negative reinforcement, so motivational factors may be important in these studies. Some behavioural tests such as eye-blink conditioning and conditioned taste aversion are respondent or classical conditioning procedures, while others such as discrimination learning and matching-to-sample are operant or instrumental procedures. Some procedures such as delayed-matching-to-sample assess short-term memory over relatively short periods of time within a test session, while other procedures such as the reference memory version of the Morris water maze assess memory over longer periods of time. Some procedures such as repeated acquisition assess responding to proximally-located experimental cues directly in the test situation, while the Morris water maze and T-maze procedures assess the ability of animals to navigate using more distally-located spatial cues. It is important to recognize that various tests for cognitive function, e.g., passive avoidance, Morris water maze, active avoidance, are assessing different forms of learning or memory and it is reasonable to expect that any one chemical may exert different effects on cognitive functioning depending on the specific test procedure used.

Methods commonly used to measure anxiety/aversion (Table 3)

48.   Anxiety is a common emotion and can be observed in laboratory rodents by typical behavioural patterns in an artificial environment. Two different types of animal models are classified: (1) Conflict is

induced between motivation (e. g. by food or water deprivation) and punishment (electrical footshock) or loss of reward. (2) Ethologically based methods are based on exploratory behaviour by creating a conflict between aversion to a situation or stimuli in novel environment (open space, height, bright illumination, unknown social partner) and the tendency to explore (Costall et al., 1988; File and Hyde, 1978; Rodgers and Cole, 1994; Silverman, 1988). Tests are simple and bidirectionally sensitive to changes of anxiety – related behaviour. Chemicals affecting motor function may interfere with the anxiety measures. Locomotor activity can be simultaneously assessed as control parameter for motor variable.

Methods Commonly Used to Measure Performance of a Complex Task (Table 4)

49.   44. Schedule-controlled operant behaviour (SCOB) involves the maintenance of behaviour (e.g., lever-press) by positive or negative reinforcement (Table 4). Different rates and patterns of responding are controlled by the relationship between response and subsequent reinforcement. SCOB provides a measure of performance and involves training and motivational variables that must be considered in evaluating the data. Agents may interact with sensory processing, motor output, motivational variables, training history, and baseline characteristics (schedule parameters). Agents may also interact with acquisition of schedule control over behaviour (eg., learning). Chemical-induced changes in stable (ie., baseline) SCOB provide insight into long-term memory and a variety of other brain functions, depending on the contingencies associated with the schedule of reinformcement (Paule 1994).

NEUROPATHOLOGY

50.   Careful consideration must be given to the purpose of a study when choosing neuropathological techniques. When chemicals are initially tested for toxicity, effective use of animals and resources involves choosing techniques that allow thorough examination of the central and peripheral nervous system, but do not disrupt pathological examination of other organs (Fix and Garman, 2000). Thus, in neurotoxicity screening (OECD 407 and 408 Guidelines), when there has been no prior indication of any neurotoxic effect, standard pathology methods involving immersion fixation and paraffin sections of nervous system tissue should be incorporated into test protocols.

51.   Immersion fixation and paraffin embedding of tissue sections are rapid, widely available techniques for screening large areas of the nervous system. These techniques are commonly used in neuropathology laboratories for tissue diagnosis and can be used with many different standard toxicology protocols. Immersion fixation is the only technique that can be used to fix tissues from animals that die spontaneously during toxicology studies. Hematoxylin and eosin stains are typically used as general stains to highlight tissue and cellular details. Often, nissl stains such as cresyl violet, are utilized for brain and nervous system tissue. Lesions present in tissues processed by these standard methods can be studied further with many different special stains and immuno-histochemical assays. The main drawbacks to using these techniques are that resolution of subtle cellular and subcellular detail is less than with plastic-embedded tissue and fixation artifacts may be more common in immersion fixed tissue as compared to perfusion fixed tissue (Cammermayer, 1960; Garman, 1990). For example, necrotic neurons produced by post-mortem hypoxia may be difficult to distinguish from necrotic neurons produced by neurotoxicant exposure.

52.   Perfusion fixation, as required in OECD Test Guideline 424, delivers fixative much more quickly to delicate internal structures than does immersion fixation. Since fixation occurs more quickly with perfusion, artifacts resulting from autolysis and from distortion of tissue during necropsy can be significantly reduced. However, perfusion itself can induce tissue artifacts particularly if intravascular pressure is excessive or pH and osmolarity are not properly controlled (Schultz and Karlsson, 1965). Perfusion can be accomplished with solutions of either formaldehyde or glutaraldehyde. Even when tissues are initially fixed using a glutaraldehyde solution, they are commonly stored in formaldehyde solutions, as glutaraldehyde tends to harden tissues with time making them difficult or impossible to

section. Glutaraldehyde-fixed tissues are not as readily used with special tissue stains as are formaldehyde-fixed tissues. The main drawbacks to perfusion fixation are that it is time consuming and usually requires adding animals to standard toxicology protocols as tissue perfusion interferes with gross pathology examinations and organ weights by changing the colour, size, and texture of organs as well as their weight. The principles and techniques associated with perfusion fixation have been detailed in several texts (Zeman and Innes, 1963; Hayat, 1970; Spencer and Schaumburg, 1980; WHO, 1986; Scallet, 1995).

53. Plastic embedding of tissues can also be used to reduce tissue artifacts and increase the resolution of cellular and subcellular detail. Epoxy and methacrylate monomers are the more commonly used media for neuropathology. An advantage to using methacrylate is that large tissue blocks can be prepared and sectioned. However, epoxy is commonly used because the same embedded specimen can be used for both light and electron microscopy. Plastic embedding can be used with either immersion-fixed or perfusion-fixed tissues. While relatively large tissue blocks can be cut from paraffin embedded tissues, plastic embedded tissue blocks are typically much smaller due to difficulties in achieving tissue penetration of plastic monomers into large tissue blocks and the difficulties associated with sectioning large blocks. Staining of plastic embedded tissues sectioned at 1µm is typically with toluidine blue; many of the special stains applicable for paraffin embedded tissues are not applicable for plastic embedded tissue.

54. Tissue selection for neuropathology studies is an important consideration. The specific areas to be examined are included in the appropriate test guideline (OECD 407, 408, 424). It may be useful to consider orienting blocks of brain tissue in the mid-sagittal plane; in this case a single section may provide representative tissue from many important structures, from the brainstem and cerebellum right on forward to the olfactory bulb. During initial neurotoxicology studies, tissue sampling should be broad so as to avoid missing an important target site in the nervous system. In addition to the tissues identified in specific guidelines, it is important for the pathologist conducting the neuropathology examinations to be aware of any information that may allow for focusing of the tissue selection and examination processes on regions of the nervous system that may be affected by a chemical exposure. The types of information that are useful for focusing the examination process include chemical structure-biological activity relationships, human case study reports, the results of prior studies, and clinical signs observed during the study. It is recommended that any nervous system tissue which is collected but not used in the initial phases of the neuropathology examination be saved as information collected during the initial slide reading may identify the need for additional focused tissue examination.

55. In conducting neuropathology examinations with experimental animals, it is important to be able to recognize spontaneous background lesions that can confound the interpretation of study results. Background lesions in the nervous system of experimental animals are common due to ageing and trauma. For a review of the more common background lesions see McMartin et al. (1997) and Mohr et al. (1994, 1996).

56. In preparing study protocols and reports of neuropathology findings, ambiguous terminology should be avoided and the nomenclature used for describing lesions and areas of the nervous system should follow agreed standards (McMartin et al., 1997). The description of lesions should include localization of the lesion to the area of the nervous system affected as well as identification of cell types involved to the degree possible. Lesion descriptions should be as specific as possible so as to accurately communicate the nature of the neurotoxic effect. Semiquantitative assessment or grading of lesions should be conducted using a defined rating scale. Attention should be paid to the distribution pattern of lesions. Chemically-induced changes typically occur in a bilateral and/or symmetrical pattern.

57. Changes in morphological appearance and staining can be deceiving (Cammermeyer, 1961). In addition, routine histology does not selectively stain degenerating neurons, making analysis more ambiguous and time consuming. Nauta and his collaborators (Nauta & Gygax, 1954) first demonstrated

how staining with ammonical silver could be suppressed so that only degenerating neurons labeled. Over the decades subsequent investigators (de Olmos et al., 1994; Nadler & Evenson, 1983; Gallays et al., 1980) developed modifications in an attempt to make the technique simpler or more reliable. Ideally, the de Olmos preparation results in the entire staining of the degenerating neuron, while the simpler Nadler & Evenson (1983) method results in a punctuate staining within cell bodies and axon terminals.

58.	Although the suppressed silver methods have the advantage of selectively staining only degenerating neurons they also tend to be labor intensive and capricious, always requiring the use of both positive and negative controls. Relatively recently two related fluorochromes, Fluoro-Jade and Fluoro-Jade B, were introduced as direct markers of neuronal degeneration (Schmued & Hopkins, 2000). Advantages include simple, rapid and reliable staining of degenerating neurons and their dendrites, axons, and terminals. The method is flexible in that, unlike suppressed silver methods, it can be used on fixed as well as unfixed tissue and can be applied to either embedded or non-embedded tissue. It is visualized with an epi-fluorescent microscope equipped with a filter system designed for visualizing fluorescein or FITC.

Specialized Neuropathology Methods for Further Lesion Characterization

59.	The methods discussed above are intended to be used to identify and characterize neuropathological lesions; therefore where applicable, these methods will be applied to tissue samples from nearly all animals on a study. When there is a need to further characterize a lesion, the examination should be focused on animals showing typical lesions in preliminary studies and on those tissues that can be expected to contain relevant lesions. Thus, the methods discussed below will typically be performed on a subset of animals in a study or on small groups of animals identified for follow-up examination.

Teased Nerve Fibre Preparations

60.	The technique of nerve fibre teasing involves separation under a dissecting microscope of individual immersion-fixed or perfusion-fixed peripheral nerve fibres. Techniques for Sudan Black staining of immersion-fixed fibres have been described by Schaeppi et al., (1984). Dyck and Lais (1970), and Spencer and Thomas (1970) have described techniques for embedding perfusion-fixed fibres in a low viscosity epoxy resin. Nerve fibre teasing is particularly useful for studying axonal and myelin lesions over the length of a fibre, identifying segmental demyelination, and studying remyelination (Spencer and Schaumburg, 1980; Griffin, 1980). Nerve fibre teasing is a labour-intensive, time-consuming process best used for studying the morphogenesis of peripheral nerve fibre lesions. When used, nerve fibre teasing should be limited to a small subset of animals showing histologic lesions and appropriate controls.

Transmission Electron Microscopy

61.	The techniques used in the preparation of ultrathin sections of the central and peripheral nervous system for transmission electron microscopy have been described by Hayat (1970), Spencer and Schaumburg (1980), and others. Due to its inherently greater resolution as compared to light microscopy, electron microscopy may be used to identify the subcellular or organellar target(s) for a neurotoxic chemical (Hirano and Llena, 1980; Thomas, 1980; Price and Griffin, 1980; WHO, 1986). Ultrastructural information may provide a basis for the formulation of an hypothesis concerning the mechanism of action for a chemical. Although electron microscopy requires extensive training, experience and capital investment (WHO, 1986), and is not a routine requirement for neurotoxicity studies, it is a powerful tool for characterizing the subcellular effects of a neurotoxicant and should be considered as an adjunct to a neurotoxicity study where other observations or information indicate it may be of value. In testing of chemicals for neurotoxicity, electron microscopy studies should be confined to those studies where there is a specific need to better characterize and more clearly define ultrastructural effects. Due to the small tissue sample size used for electron microscopy, proper tissue selection is absolutely essential to ensure that

changes which are observed by light microscopy are selected for ultrastructural examination. Electron microscopy is considered to be a technique which is to be used in addition to and in support of light microscopy.

Morphometric Methods

62.   Stereological methods for quantification of total number of neurons or other cell types, volume of brain regions, lengths of structures, surface areas, and size of neurons or neuronal nuclei have been published (Gundersen et al., 1988 a, b; Korbo et al., 1990, 1993; Coggelshall and Lekan, 1996; Geinisman et al., 1997; Hyman et al., 1998; Peterson, 1999; Schmitz, 1997; Skoglund et al., 1996). These methods avoid certain types of sampling bias as they are not based on an assumption about the shape of the tissues or the structures found in the tissue. Neuronal losses in rat brains as low as 16% have been detected with stereological methods (Strange et al. 1991; Korbo et al. 1996). Efficient, unbiased sampling techniques are required to maximize the usefulness of stereological methods. In particular, where acute evaluation using degeneration-selective methods has failed to reveal neurotoxicity, the application of morphometric methods following chronic exposure may be indicated. These techniques are sensitive to more gradual changes in such parameters as the number of neurons, as well as the number and shape of their dendrites, synapes, etc., that may mark the cumulative effects of certain neurotoxicants (Scallet, 1995).

Morphological Methods Based on Immunohistochemistry

63.   Immunohistochemical methods have been used to better characterize findings seen or observed with general stains. In some cases immunohistochemical methods can be used to demonstrate processes that precede observable tissue damage. Due to their ability to localize changes in the nervous system, immunohistochemical methods can be superior to biochemical methods for detecting subtle lesions. Morphological staining techniques have been adapted from biochemical and immunological analytical methods. For example, enzymatic methods can be used to histochemically visualize the loss of capacity to generate energy following mitochondrial neurotoxicity (Nony et al., 1999). There are numerous peptides in the nervous system that may potentially be demonstrated by immunohistochemistry during neurotoxicological processes. For example, the "heat shock" protein, ubiquitin, is expressed during pathological processes in a variety of cells including those of the nervous system (Mayer and Westbrook, 1987). Certain laboratories have used batteries of immunohistochemistry and tissue radio-immunoassay tests for selected neurotypic or gliotypic proteins to detect changes induced in the nervous system by a limited number of neurotoxic chemicals, particularly trimethyl- and triethyltin (Brock and O'Callaghan,1987; O'Callaghan and Miller, 1988).

NEUROPHYSIOLOGY

64.   Electrophysiological techniques are commonly used by neurophysiologists and clinical neurologists. Thus the experimental and clinical literature provide a large body of background information to assist interpretation of test data (Thompson and Patterson, 1974; Barber, 1980). The neural basis of most electrophysiological tests is known, and many of them can be readily applied across species, including man. The latter points, facilitate their interpretation and extrapolation to man. Nevertheless, a multidisciplinary approach (e.g., including behavioural and neuropathological endpoints in the study) is recommended to facilitate a better understanding of the effects of chemicals on the nervous system (WHO, 1986; OTA, 1990).

65.   The heartbeat dynamics (as measured by R-R signal from ECG) was demonstrated to be a very efficient probe of the status of the autonomic nervous system (Akselrod, 1988) both for humans (Mestivier et al., 1997; Giuliani et al., 1998) and in experimental animals (Japundzic et al., 1990; Dabiré et al., 1998). Also in epidemiological studies, the sensitivity of the heart rate dynamics measures as a probe of

neurotoxicity was demonstrated to outperform that of any other observable physiological marker (Murata et al., 1994; Araki et al., 1997; Arden et al., 1999; Liao et al., 1999). These features, together with the common meaning of heartbeat dynamics across species and the relative ease of the proposed measurement, make heart rate variability (HRV) a good candidate for neurotoxicity testing.

66.   Some neurophysiological techniques can be resource intensive (requiring both specialist equipment and trained staff) and may not be commonly available at contract laboratories. In addition, some electrophysiological tests are invasive in animals and may require satellite groups if they are to be included in a toxicity study.

Electroencephalography

67.   The frequency, amplitude, variability and pattern of the EEG is a measure of the dynamic process of the instantaneously integrated activity of the brain (see Table 5).   The EEG reflects instantaneous changes in the state of CNS activity, and, thus, is often used as a measure of the state of arousal or anaesthesia.

68.   An EEG obtained using surface electrodes or epidural electrodes is a summation of the electrical activity mainly from neurons a few millimetres beneath the electrode and may not accurately reflect activity of deeper structures.   By implanting electrodes into subcortical structures their activity can be recorded (Dyer et al., 1979a, b; Naasland, 1986).   The EEG cannot provide information on the integrity of specific motor or sensory pathways.   The data produced can be difficult to interpret and, as it does not provide information at the cellular level, it may be difficult to use to provide details on mechanism of toxicity (Johnson 1980; Arezzo et al. 1985; Seppalainen 1988).

Evoked Potentials (EPs)

69.   Electrical potentials recorded from the brain in response to external stimuli are called evoked potentials (EPs). The types of EPs most commonly recorded in experimental animals are listed in Table 5.

70.   Visually evoked potentials (VEPs) include flash evoked potentials (FEPs) and pattern evoked potentials (PEPs) and are used to evaluate the effects of substances on the components of the nervous system responsible for vision. Potentials can be generated using stimuli ranging from diffuse light flashes to complex patterns of shape and colour. If abnormalities in FEP are observed, electroretinograms may be used to aid in the interpretation (Rebert, 1983). PEPs are indicators of the ability to perceive patterns, and can be elicited by shifting patterns on a television screen or computer monitor. Their testing requires proper accommodation of the stimulus on the retina, and fixation of the awake animal in front of the screen.

71.   Auditory EPs may be recorded from the cortex or the brainstem (BAER) in response to brief auditory stimuli (clicks or pips) and can be used to detect specific losses in the auditory system. The cortical auditory EPs require awake animals.   The BAER can be obtained also with subcutaneous electrodes above the cerebellum and the brain stem in awake restrained or anaesthetised animals.

72.   Somatosensory evoked potentials (SEPs) are elicited by electrical stimulation of sensory receptors or peripheral nerves at the foot, tail or skin and are recorded from the somatosensory cortex. Recording from the cerebellum can help to differentiate effects and/or more precisely localise lesions. SEPs, like other evoked potentials, are not without interpretational difficulties as they may be altered by a variety of factors such as toxic reactions, temperature, hypoxia, sensory deficits, central dysfunction, vitamin deficiency, or state of surroundings of nerve tract (Rebert, 1983; Albee et al, 1987, Sohmer 1991). It may sometimes be difficult, therefore, to discriminate a direct neurotoxic effect from other consequences of treatment.

Peripheral Nerve Conduction Velocity

73.　　The nerve conduction velocity (NCV) is one of the most commonly used electrophysiological tests for neurotoxicology studies. Electrophysiological tests may be performed in vitro on excised nerves (Birren and Wall, 1956) or in vivo using surgically exposed nerves (McDonald, 1963; DeJesus et al, 1978) or in the intact animal (Table 6). Another approach to measure the peripheral nerve conduction velocity is the recording of the H-reflex (Stanley 1981; Mattsson et al. 1984). The NCV of motor and sensory nerves can be determined separately.

74.　　The nerve segment being evaluated must be long enough to permit accurate measurement so that not all nerves can be easily evaluated in rodents. It is important to correct the calculated conduction velocity for variations in the temperature in the tissue or bath surrounding the nerve (Birren and Wall, 1956; DeJong et al, 1966).

Electromyography (EMG)

75.　　The electromyogram (EMG) is an extracellular recording of the electrical potential of a striated muscle obtained from surface electrodes or by inserting needle electrodes through the skin into the muscle. It has been used extensively in human clinical studies in the diagnosis of certain myopathies and neuropathies (Licht, 1961; Merletti et al. 1992) but only occasionally in neurotoxicity studies involving experimental animals (Table 6). One of the reasons for the limited use of the EMG in toxicology is that one component of the EMG involves the voluntary graded contraction of the muscle being evaluated which can be difficult in laboratory animals.

Single Cell Recordings and Ex Vivo Techniques

76.　　Electrophysiological techniques such as intracellular microelectrode recording, iontophoresis and voltage or patch clamp enable cellular mechanisms of action of neurotoxicants to be determined (see Table 3). These techniques (e.g., patch clamping for the investigation of individual ion channels), have been reviewed extensively by Kerkut and Heal (1981), Atchison (1988), and Baxter and Byrne (1991).

NEUROCHEMISTRY: NEUROCHEMICAL METHODS

77.　　Normal functioning of the nervous system requires that the many biochemical mechanisms that underlie nerve signal propagation, synaptic transmission and plasticity operate properly. To assess the functional integrity of neurons or glial cells a multitude of biochemical or neurochemical endpoints may be evaluated in neurotoxicity studies. Among these endpoints, some provide information about the presence or abundance of specific cells, others are related to the integrity of cells or to their function. The value of these endpoints is best seen where they can be correlated with other endpoints of neurotoxicity, as e.g. structural or behavioural changes or where they are related to the mechanism of toxicity. Due to the complexity of the nervous system with its myriad of chemical messengers, selective use of neurochemical methods as a primary test may be warranted but only under some circumstances. The strength of neurochemical methods, including both in vivo and in vitro approaches, lies in helping to identify the primary target site and in defining the mechanism of action for neurotoxicants, or in rapidly screening for a known or suspected mechanism of toxicity. One such example is the synthesis of heat shock proteins in the cells in response to an insult by a neurotoxic agent MPTP (Freyaldenhoven and Ali, 1995; 1996; Freyaldenhoven et al, 1997).

General Biochemistry

78.　　General biochemical attributes of nerve tissue can be used to determine the integrity of neurons or glial cells. Such general biochemical measures include endpoints of cellular toxicity, changes in energy-

linked functions or in synthesis of cell constituents or proteins. For endpoints related to cell death generally accepted criteria for adverse effects exist while for the other endpoints criteria are problematic and should be correlated with other neurotoxic endpoints.

79.   An example of an endpoint related to cell constituents is myelin which is responsible for most of the lipid content of neural tissue and contains unique proteins such as myelin basic protein. Changes in lipid composition can reflect demyelination; such changes may consist of an increase in water content accompanied by a decrease in myelin protein/lipid and can be accompanied by the appearance of cholesterol esters (Norton and Cammer, 1984). Similarly, myelin basic protein content or synthesis (Hamano et al., 1996; Conti et al., 1996) may be determined to quantify myelination in the brain or reactive synthesis secondary to a toxic insult. Heat shock proteins are synthesized in cells in response to a variety of stresses including exposure to neurotoxic compounds (Gonzalez et al., 1989). Where the mechanism of neurotoxicity is known and a relevant biochemical endpoint can be defined, direct measurement of this endpoint may provide a sensitive and reliable estimate for neurotoxicity. An example for a mechanistic-based neurochemical endpoint is neuropathy target esterase (Abou-Donia and Lapadula, 1990) which can be inhibited by a sub-set of cholinesterase-inhibiting chemicals and has been associated with organophosphate-induced delayed neuropathy. Decreases in mRNA or protein synthesis (Schotman et al., 1978; Albrecht, 1984), changes in energy-linked functions (Lai et al., 1980; Husain et al., 1986; Sickles and Goldstein, 1986) or increased production of oxygen radicals (LeBel et al., 1990) may provide evidence for a neurotoxic effect. Changes in these parameters may also be induced by pharmacologically active chemicals (Muller and Martin, 1984), during sensory stimulation (Sharp et al., 1981) altered activity or emotion (Roland et al., 1982; LeDoux et al., 1983) and should be differentiated from those related to toxicity.

Cell-specific markers

80.   Quantification of cell specific markers can be helpful in identifying cell degeneration or reactive gliosis, but in general are poor predictors of the functional integrity of cells. Methods used to quantify specific cells may best be correlated with histopathology or antibodies directed toward such markers may be used to immunohistochemically localize morphological changes (see also Neuropathology section). Selection of the appropriate marker and the relevant time for its determination is crucial in order to see an effect.

81.   Glial Fibrillary Acidic Protein (GFAP), the major intermediate filament protein in astrocytes has been shown to increase as a result of chemical (e.g., cadmium, MPTP) induced nervous system injury that produces hypertrophy of astrocytes (O'Callaghan, 1991) and can be measured using immunoassay or immunohistochemical methods. Changes in several of these cell type specific markers e.g., c-fos or fra-related antigens can also be induced by pharmacologically active chemicals that do not cause neurotoxicity (Murphy et al., 1991), while changes in others are transient (Sakurai-Yamashita et al., 1991) and may be missed when measured outside of the relevant time window.

Neurotransmission

82.   Neurotoxicants can perturb neurotransmission or ion channel function in neurons with either short-term or more persistent effects, as seen with other neurotoxic measures. Biosynthetic enzymes, uptake mechanisms, storage, release or degradation of the neurotransmitter, receptor function, signal transduction etc. may be affected. This may then lead to changes in neurotransmitter concentration or its metabolites or to up- or down-regulation of receptors. The toxicological interpretation of such neurochemical effects (especially transient ones) can be problematic as they may also occur as the result of a pharmacological effect. Therefore, the value of neurochemical indicators is best seen where they can be correlated with other neurotoxic endpoints, such as behavioural changes.

83.   Rather than attempt to cover the diverse and expanding list of chemical neurotransmitter channels in this Guidance Document, the use of neurochemical methods as a tool in neurotoxicity evaluation will be illustrated by reference to a representative neurotransmitter, acetylcholine. Recent publications (e.g., Siegel et al., 1998) should be referenced for an up-to-date listing of identified neurotransmitters that may be investigated in neurochemical evaluation of toxicants.

Acetylcholine example

84.   Neurotransmitter or precursors uptake can be measured both *in situ* at the synapse or in *ex situ* models for the presynaptic nerve terminal such as synaptosome preparations (Lai et al., 1980). Neurotransmitter concentrations can be measured directly in neural tissue using techniques such as microdialysis (Takahashi et al., 1998).

85.   Release of transmitter can be measured from synaptosomes, tissue slices (Login et al., 1998) or in situ (Schilstrom et al., 1998). Neurotransmitter receptors exhibit a number of different subtypes having specific pharmacological characteristics and unique nervous system distributions. Using labelled neurotransmitter or neuromodulator ligands, receptor characteristics can be measured in vitro using purified membrane fractions, or by autoradiography on thin tissue slices. Ex vivo or in vivo studies can extend information about the distribution and regulation of receptors in different brain regions (Nakayama et al., 1997, Flynn et al., 1997). Pharmacological analysis using techniques such as Scatchard analysis (Scatchard, 1949) allow such parameters as dissociation constant and receptor density to be characterized. Chemicals acting at the receptor level can produce transient or persistent effects. Up- and down-regulation of receptors is a common property of many neurotransmitter receptors and can lead to prolonged effects, outlasting exposure to the test chemical. (Miao et al., 1998, Pope et al., 1992).

86.   Acute or repeated exposures may result in different neurochemical effects, especially when critical periods for nervous system development are considered (Chanda and Pope, 1996). Subsequent events in the signal transduction pathway such as second messengers, protein phosphorylation or gene activation can also be evaluated as neurochemical endpoints (Costa, 1992; Zimmer et al., 1997). For most transmitters, inactivation is achieved through an uptake mechanism at the plasma membrane. In the case of acetylcholine, acetylcholinesterase enzyme located on the post-synaptic surface hydrolyses the transmitter to acetic acid and choline. Acetylcholine accumulation resulting from acetylcholinesterase inhibition can be manifest in a number of ways such as down-regulation of muscarinic receptors, an effect that may contribute to tolerance, cognitive dysfunction or heightened sensitivity to cholinomimetics (Pope et al.,1992); choline itself can have significant agonist activity at certain acetylcholine receptor subtypes.

87.   The target sites for neurotoxic action illustrated with acetylcholine (uptake, receptor binding, inactivation) apply also to other chemical neurotransmitters. Other neurotransmitters that have been the subject of extensive research include dopamine. The nigrostriatal dopaminergic system is affected by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is converted to the active toxicant 1-methyl 4-phenylpyridinium ion (MPP+) by the enzyme, monoamine oxidase B. MPP+ is a substrate for the neostriatal dopamine uptake system (Javitch et al., 1985); consistent with this mechanism, dopamine uptake inhibitors and monoamine oxidase B inhibitors are effective protectants against tetrahydropyridine neurotoxins. Amino acid transmitter receptors have been identified as the primary target for some neurotoxicants (e.g. kainic and domoic acid). Neurotoxic effects can be produced by excessive activation or excessive inhibition of specific excitatory amino acid receptors (Olney, 1995; Tilson and Mundy, 1995) and have been extensively correlated with neuropathology.

88.   Nerve membrane voltage-sensitive ion channels are also potential targets for neurotoxicity. At the sodium channel as an example, toxins can act through blockade (tetrodotoxin), activation (batrachotoxin), or a change in voltage sensitivity/inactivation (DDT). Binding and localization studies using labelled

toxins can be conducted on ion channels. Disruption of ion channel function is often best studied by electrophysiological methods.

In vitro

89.   In vitro techniques for neurotoxicity assessment is a rapidly evolving field of research and a recent, comprehensive review should be consulted (Harry et al., 1998).

90.   Neurochemical approaches to neurotoxicant evaluation make extensive use of in vitro and molecular biology techniques. For specific receptor subtypes, cloned receptors can be transiently expressed in oocytes or stably expressed in non-neuronal cell lines (Gopalakrishnan et al., 1996) and receptor selectivity (Yamamoto et al., 1998) is one useful criterion in neurotoxic risk. Neurotransmitter uptake transporters and ion channels have been cloned and are available for expression in vitro. Studies of neurotransmitter release can also be investigated in cell cultures using clonal cell lines (Greene & Rein, 1977). In addition to studies with clonal cell lines, transmitter function can also be studied in primary culture using fetal rat neural tissue: examples include nicotine-stimulated catecholamine release from CNS neurons (Gallardo and Leslie, 1998) or neurotoxicant effects on cholinergic stimulated second messenger metabolism (Kovacs et al, 1995). Explants of brain tissue offer an additional level of organizational complexity, retaining aspects of the structural and functional characteristics of the source tissue, though with the attendant limitations resulting from denervation produced by the explant procedure.

91.   It is not possible to set up and operate specialized in vitro tests for all likely neurotoxic mechanisms. Nevertheless, several mechanism-independent in vitro neurotoxicity models have been proposed and are under further investigation (Atterwill et al., 1994). Various factors can contribute to either an overestimation or underestimation of the extent to which a particular compound poses a risk to the whole organism. Examples of these factors are the absence of blood-brain barrier, diminished or absent metabolic capacity of in vitro preparations or difficulties in extrapolating concentration of the chemical in the culture medium to exposure levels. The subtle effects of neurotoxicants on certain integrative functions of the nervous system, such as memory, learning, and sensory processing cannot be predicted from in vitro data.

Table 1: Tests Commonly Used to Measure Motor Function

Test	Measurement	Agents	Species	Comments	References
Grip Strength (See OECD 407, 408, 422, 424)	Muscular strength in fore and hindlimbs using strain gauges	Decreased by agents that produce peripheral neuropathy (eg., acrylamide); increased by agents that produce hypertonia (eg., 2,4,-D)	Used with rats and mice; it may be difficult obtaining grip strength, especially hindlimb grip strength in young (pre-wean) animals.	Training of animals not required; animals may not grasp if severely impaired. May be influenced by body weight. Some equipment required	Broxup et al., 1989 Meyer et al., 1979 Moser & MacPhail, 1990 Pryor et al., 1983 Schulze & Boysen, 1991 Tilson & Moser, 1992 Ross, 2001
Rotating Rod (Rotorod, Accelerod)	Animal is placed on the circumference of a rotating rod. Measure the time to fall off, or the speed of rotation when the animal falls off. Provides measure of motor function/co-ordination.	Decreased by agents that produce peripheral neuropathy (eg., acrylamide), ataxia (eg., ethanol), or vestibular dysfunction (eg., IDPN)	Rats and mice; can be used in pre-weaned rats and mice	Little training of animals is required Animals may "voluntarily" jump off Equipment required Compatible with standard toxicology studies	Alder & Zbinden, 1983 Bogo et al., 1981 Capacio et al., 1992 Kaplan & Murphy, 1972
Hindlimb Foot Splay Landing Foot Spread	Measure distance between feet; to assess neurological response following loss of vertical support	Increased by agents producing peripheral neuropathy (eg., acrylamide)	Adult rats	Decreases may be difficult to interpret Training of animals not required Equipment is minimal Compatible with standard toxicity studies	Broxup et al., 1989 Edwards &Parker, 1977 Moser et al., 1988 Moser & MacPhail, 1990 Tilson & Moser, 1992 Ross, 2001

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Table 1: Tests Commonly Used to Measure Motor Function (Cont'd)

Test	Measurement	Agents	Species	Comments	References
Motor Activity (Used in OECD 407, 408, 422, 424)	Measures horizontal and/or vertical movements in a test chamber	Increased by CNS stimulants (eg., amphetamine), and muscarinic receptor antagonists (eg., scopolamine). Decreased by CNS depressants (eg., chlorpromazine), cholinesterase inhibitors (eg., carbaryl). Biphasic dose-response effects with some chemicals, i.e., increases at low doses and decreases at higher doses (eg., pentobarbital, solvents)	. Rats 13 days or older; if testing younger animals, the apparatus may have to be modified to obtain values comparable to adults Adult mice	Automated equipment is necessary Training of animals is not required May be influenced by competing motor behaviours (eg., stereotypy, freezing), especially when induced pharmacologicallly	Archer, 1973 Alder & Zbinden, 1983 Broxup et al., 1989 Crofton et al., 1991 Pryor et al., 1983 Reiter, 1978 Ruppert et al., 1982 Schorring, 1979 Schulze & Boysen, 1991 Maurissen, & Mattsson, 1989 Ruppert et al., 1985

Table 2: Tests Commonly Used to Measure Sensory Function

Test	Measurement	Agents	Species	Comments	References
Nociception (Hot Plate, Tail Flick)	Subject is placed on a warm surface and latency to lift or lick a paw is recorded. Time required for animal to move tail in response to mechanical or thermal stimulus applied to tail.	Latency is increased by analgesics (eg., opiates), cholinesterase inhibitors (eg., carbaryl), and some neurotoxicants (eg., methylmercury)	Adult mice and rats Neonatal rat	Training of animals not required. Some equipment necessary Skin temperature or core temperature could affect result May not be suitable for frequent repeated testing Compatible with standard toxicity studies	Pryor et al., 1983 Walsh et al., 1984a Takagi et al., 1966 McCormack et al., 1998 Hole And Tholsen, 1993
Sensory Irritation	A small volume of mild irritant solution (e.g., zingerone) is placed on the eye, and the number of "wipes" or the duration of wiping is measured	Capsaicin Acrylamide	Adult rat	Training of animals not required Little or no equipment is required Compatible with standard toxicity studies	Miller et al., 1984 Szolcsanyi and Jansco-Gabor, 1975
Somatosensory Operant Discrimination Task	Animals are trained to respond in the presence of a stimulus cue (eg., light, noise, odour). Measure the ability of animal to discriminate changes in the cue.	Chemicals affecting peripheral nerve function (eg., acrylamide), nociception (eg., cholinesterase inhibitors, or heavy metals triethyl tin)	Adult rats, monkeys	Extensive training required May require motivational manipulation (e.g., food or water deprivation) to maintain response Requires equipment to control stimuli and record responses Allows repeated testing of same animals during repeated dosing Sensory assessment may be confounded by effects of chemicals on motor function Satellite groups required Not compatible with standard toxicity testing	Elsner, 1991 Maurissen et al., 1983 Tilson & Burne, 1981 Weiss & Laties, 1961 Wood, 1979; 1981

Table 2: Tests Commonly Used to Measure Sensory Function (Cont'd)

Test	Measurement	Agents	Species	Comments	References
Acoustic Startle Response and Prepulse Inhibition	The magnitude and latency of the reflex response to a auditory stimulus is measured. Prepulse inhibition version of the test includes presentation of a brief sound stimulus prior to the reflex-eliciting eliciting stimulus. Prepulse stimuli inhibit the startle reflex. Because prepulse inhibition depends on the ability to perceive the inhibiting stimulus, varying prepulse intensity or frequency can be used determine auditory thresholds.	Increased responsiveness (eg., pyrethrin, DDT). Agents causing ototoxicity (solvents and certain antibiotics) can decrease responsiveness and increase threshold for hearing. Agents causing CNS depression (eg., pentobarbital) may produce non-specific decreases in reactivity.	Adult rats, mice	Requires multiple presentation of stimuli and recording of responses Permits retesting of same animals Requires little training of animals Requires equipment to present stimuli and record responses Startle response is a motor response and interpretation of results as a deficit in sensory function could be problematic without appropriate controls Prepulse inhibition procedure includes control for motor component Prepulse inhibition procedure allows for testing of multiple sensory modalities Could be used with standard testing guideline studies, but scheduling could be difficult Startle magnitude may be influenced by body weight Animals usually restrained during startle testing and this may affect neurochemical and endocrine measures.	Chiba and Ando, 1976 Crofton & Reiter, 1984 Crofton & Sheets, 1989 Fechter & Young, 1983 Hoffman & Ison, 1980 Ison & Hoffman, 1983 Marsh et al., 1978 Pryor et al., 1983 Wecker & Ison, 1984 Young and Fechter, 1983 Davis et al., 1982a Davis, 1980 Crofton, 1992

Table 2: Tests Commonly Used to Measure Sensory Function (Cont'd.)

Test	Measurement	Agents	Species	Comments	References
Auditory Discrimination Procedure	Animal is trained to make a response to obtain reinforcement in the presence of an auditory cue. Measure the accuracy of responding when the cue is modulated experimentally. Chemical exposure alters perception of cue and impairs accuracy of responding.	Ototoxic agents (eg., antibiotics) impair accurracy	Adult rats, guinea pigs, monkeys	Requires extensive training of animals to learn discrimination Often requires food or water deprivation to motivate responding Requires equipment to present stimuli and record responses Permits repeated testing of same animals during repeated dosing Permits determination of thresholds Sensory assessment may be confounded by effects of chemical on motor function Satellite groups required Not compatible with standard toxicity studies	Stebbins & Rudy, 1978 Bushnell et al., 1994 Pryor et al., 1987
Visual Discrimination Task	Animal is trained to make a response to obtain reinforcement in the presence of a visual cue, which can be modulated experimentally. Chemical exposure alters perception of cue and impairs accuracy of responding	Decreased by psychotropic agents (eg., hallucinogens), sensory toxicants (eg., methylmercury)	Adult rats monkeys, pigeons	Requires extensive training to learn discrimination Requires food or water deprivation to motivate responding Requires equipment to present stimuli and record responses Permits repeated testing of same animals during repeated dosing Permits determination of thresholds Rats are generally not a good model for visual dysfunction assessments Sensory assessment may be confounded by effects of chemical on motor function Satellite groups required Not compatible with standard toxicity studies	Blough, 1957 Evans et al., 1975 Friedman & Carey, 1978 Merigan et al., 1982 Rice & Gilbert, 1982

Table 3: Tests Commonly Used to Measure Cognitive Functions

Test	Measurement	Agents	Species	Comments	References
Habituation	A gradual decline in the magnitude or frequency of a response after repeated presentation of a discrete stimulus (e.g., diminished startle response or orienting response to an acoustic stimulus) or complex stimulus (e.g., decrease in motor activity, rearing, head dips in a test chamber as a function of time). Can be assessed within or between sessions.	Cholinesterase inhibitors, organochlorine insecticides (eg., pyrethrin), lead, dietary iron, fungizides	PND 21 rats, adult rats, mice	Training of animals not required Equipment to perform motor activity or acoustic startle is required Reactivity in test condition could be influenced by non-cognitive variables Interpretation of data may depend on operational definition of when habituation has occurred Within-session habituation occurs in motor activity testing in guideline studies Assessment of inter-session habituation would be difficult in guideline studies	File and Wardill, 1975 Gerhardt et al., 1993 Overstreet, 1977 Ruppert et al., 1985 File, 1981 Ross, 2001 Pilz and Schnitzler, 1996 Davis, 1982b Buelke-Sam et al, 1985
Ethologically based anxiety tests (Elevated plus maze test, Black and white box test, Social interaction test)	Animal is placed in a novel aversive environment (apparatus with two open and two enclosed arms in height; a two compartment box with one compartment dark and one brightly illuminated; in a box with an unknown partner). Measures number of entries and time spent in the aversive parts and contact frequency and time, respectively, locomotor activity	Lindane, sarin, soman, TMPP, parathionmethyl, nicotine receptor agonists, methyl mercury, scopolamine	Rats, mice, guinea pigs	Tests are simple, rapid, require no training, minimal equipment Food deprivation not required Use of noxious stimuli not required Testa are bidirectionally sensitive	Costall et al., 1988; File and Hyde, 1978; Rodgers and Cole, 1994; Silverman, 1988

Table 3: Tests Commonly Used to Measure Cognitive Functions (Cont'd)

Test	Measurement	Agents	Species	Comments	References
Conditioned Taste Aversion (CTA)	A decrease in intake of a food or fluid following pairing of the food or fluid with chemical exposure. Measure extinction of CTA to evaluate learning. Can measure threshold for taste avoidance of food or fluid to assess gustatory neural sensitivity.	Agents producing non-specific illness (eg., lithium chloride), psychoactive agents (eg., amphetamine, triadimefon), neurotoxicants (eg., trialkytins, IDPN, capsaicin)	Primarily adult rats but can be used in most species	Response measurement (i.e., change in intake), is relatively simple. Little equipment required. Delays can be introduced between pairing of stimuli to assess -memory. Few learning trials requireTest chemical can be used as unconditioned stimulus (UCS) for CTA or the influence of the test chemical on development of CTA to another agent (eg., lithium) can be evaluated. CTA can be used to measure the degree of aversion as an index of toxicity, rather than a measure of learning. Usually requires food or water deprivation. Satellite groups required. Not compatible with standard toxicity studies	MacPhail, 1982 Peele et al., 1990 Peele, 1989 Riley &Tuck, 1985
Active Avoidance	Animal is placed in one chambers and exposed to a cue (eg., light, tone) that precedes a negative reinforcer. Animals can avoid exposure to the negative reinforcer by responding to the cue that precedes the negative reinforcer. Learning is measured as the frequency and latency to avoid the negative reinforcer following presentation of the cue	Increased trials to learning criterion (eg., 80% correct) from agents that impair somato-sensory function (eg., chlorpromazine), organochlorine insecticides (eg., chlordecone, DDT, lindane)	Adult rats and mice	Animals are given repeated trials and learning occurs within session (massed trials) or over several days (discrete trials). Requires negative reinforcement. Sensitivity to reinforcer may be altered by test agent. Requires prominent sensory and motor component, which must be considered in interpretation of data. Requires equipment to present stimuli and record responses. Sensitive to effects on learning or acquisition, but sensitivity as measure of performance has not been well established. Agents that alter motor activity may non-specifically affect performance of this task. Not compatible with standard toxicity studies. Satellite groups required	King, 1985 Tilson et al., 1982

Table 3: Tests Commonly Used to Measure Cognitive Functions (Cont'd)

Test	Measurement	Agents	Species	Comments	References
Passive Avoidance	Animals are taught to withhold a response following pairing of an cue with the presentation of a negative reinforcer. Response is measured as frequency and latency to avoid a negative reinforcer by not making an active response following presentation of the cue that predicts the negative reinforcer (ie., avoiding the negative reinforcer requires that no response is made)	Decreasing latency to respond after muscarinic receptor antagonists (eg., scopolamine), organochlorine insecticides (eg., chlordecone, trialkyltins)	Weanling or older rats and mice	Requires few training trials to observe learning May require additional test sessions to demonstrate retention Requires negative reinforcement Sensitivity to reinforcer may be altered by agent Requires prominent sensory and motor component Agents that non-specifically alter motor activity could interfere with performance of this task Requires equipment to present stimuli and record responses Could be used with guideline protocols	Costa & Murphy, 1982 Mactutus et al., 1982 Peele et al., 1990 Walsh et al., 1982b
Spatial Mazes (Morris water maze, Biel water maze, T-maze)	Animals are taught to navigate a maze in order to obtain positive reinforcement (eg., food) or escape negative reinforcement (eg., escape from water). Measure is time to obtain reinforcement, accuracy of responding, time to escape, response accuracy.	Trimethyltin, excitatory amino acid neurotoxicants (eg., domoic acid), cholinesterase inhibitors	Adult rats and mice	Animals are trained over several days to learn task Requires motivational manipulation for positive or negative reinforcement; Requires food or water deprivation to motivate responding if food or water positive reinforcement used Permits repeated testing of same animals during repeated dosing Requires prominent sensory and motor component Sensitivity to reinforcers may be altered by agent Generally rRequires equipment to present stimuli and record responses Once learning has occurred, procedure allows for determination of various forms of memory (e.g., working, reference), and ability to perform cued discriminations Not compatible with standard toxicity studies Satellite groups required	Alessandri et al., 1994a,b Bushnell & Angell, 1992 Chrobak et al., 1987 FitzGerald et al., 1988 McDonald et al., 1988 Milgram et al., 1988 Petrie et al., 1991 Rogers & Tilson, 1990 Walsh and Chrobak, 1987 Walsh et al., 1984b D'Hooge and De Deyn, 2001

Table 3: Tests Commonly Used to Measure Cognitive Functions (Cont'd)

Test	Measurement	Agents	Species	Comments	References
Conditional Discrimination (simple discrimination, matching to sample)	Animals are trained to perform a response for reinforcement in the presence of a cue such as a light or tone Cues may be presented to guide next opportunity for reinforced response Response contingencies that predict the availability of reinforcement may change on a regular basis, e.g., daily, requiring learning within session. Considered a measure of learning, stimulus control is assessed by training animals to make separate responses to different stimuli. Rate, accuracy and patterns of responding can be measured	Lead, cadmium, carbon monoxide	Rats, monkeys, pigeons	Requires extensive training over daysMotivational manipulation needed to maintain responding Usually requires food or water deprivation to motivate responding Equipment to present stimuli and record responses is required Requires prominent sensory and motor component Modality and intensity of stimuli, as well as motivation and behavioural response can affect outcome. Permits repeated testing of same animals during repeated dosing Not compatible with standard toxicity studies. Satellite groups required	Gabriel et al., 1991 Heise & Millar, 1984 McMillan, 1981 Paule et al. 1998 Paule, 2000 Rice & Gilbert, 1990 Rice, 1984; 1985 Schrot et al., 1984 Winneke et al., 1977 Zenick et al., 1978
Delayed Discrimination (delayed matching-to-sample, delayed alternation) Repeated Acquisition	Similar to conditioned discrimination but is a measure of learning and memory. Retention is assessed by measuring accuracy of performance following a delay between presentation of a discriminative stimulus and the opportunity to respond	Trimethyl tin, psychotropic drugs, cholinesterase inhibitors	Rats, monkeys, pigeons	Same as for conditional discrimination Requires food or water deprivation to motivate responding if food or water positive reinforcement used Not compatible with standard toxicity studies Satellite groups required	Dews, 1971 Katz, 1982 Evans et al, 1975 Paule, 1994, 1995, 2000 Cohn and Paule, 1995 Schrot et al., 1984 Rice, 1984; 1985 Eckerman and Bushnell, 1992

Table 3: Tests Commonly Used to Measure Cognitive Functions (contd.)

Test	Measurement	Agents	Species	Comments	References
Eye-Blink Conditioning	A stimulus such as a tone is paired with another stimulus (e.g., air puff or small electric shock) that elicits an eye-blink response. After a number of trials the tone elicits a conditioned eye-blink response. Measure the trials to produce the conditioned eye-blink response and the amplitude and latency of the conditioned response is measured.	tetrahydrocannabinol, MAMM, anticholinergic agents, heavy metals, ethanol.	Rats, monkeys	Electrode is implanted to record eyelid activity and administer eliciting stimulus. Learning can occur with repeated trials in single day. Does not require motivational variables. Requires equipment to present stimuli and record responses. Does not require prominent sensory and motor component to perform. Not compatible with standard toxicity studies. Satellite groups required	Stanton & Freeman, 1994

Table 4: Tests Commonly Used to Measure Performance of a Complex Task

Test	Measurement	Agents	Species	Comments	References
Schedule- Controlled Operant Behaviour (SCOB)	Animals are trained to make response (eg., press a lever) to obtain positive reinforcement. The pattern and rate of responding, which are controlled by the response-reinforcement relationship, are measured.	Methylmercury, pesticides, acrylamide, carbon monoxide, lead, solvents, tins	Adult rats, mice, monkeys	Requires training to obtain terminal baseline of responding Requires motivational variables to maintain responding Requires food or water deprivation to motivate responding if food or water positive reinforcement used Requires prominent sensory and motor component that should be considered in the interpretation of the data Requires equipment to present stimuli and record responses Permits repeated testing of same animals during repeated dosing Provides stable baseline of performance to study agents that affect the nervous system Satellite groups required	Anger et al., 1979 Armstrong et al., 1963 Barthalmus et al., 1977 Rees and Li, 1994 Cory-Slechta et al., 1981 Dietz et al., 1978 Laties & Evans, 1980 MacPhail & Leander, 1981 Moser & MacPhail, 1990 Paule, 1994, 1995, 2000 Rice, 1988 Tilson et al., 1980 Weiss et al., 1981 Wenger et al., 1984 Li et al., 1999 Moser et al., 2000

Table 5: Tests Which Evaluate the Spontaneous and Evoked Electrical Activity of the Brain

Test	Measurement	Agents	Species	Comments	References
Spontaneous Potentials Electro-encephalography (EEG)	Measure spontaneous electrical activity from the most superficial layers of brain (cortex) Stages of wake / sleep and seizure activity can be readily observed	Anaesthetics, toluene, organophosphates	Most laboratory animals Usually implanted electrodes are used in rodents Awake dogs can be tested with removable sub-dermal pin electrodes	Only major changes are directly visible Quantitative analysis of data is necessary to find less obvious changes in frequency, amplitude, variability or pattern Not compatible with standard toxicity studies (except dog studies) Satellite groups required	Beningnus, 1969; 1984 Beningnus & Muller, 1982 Eccles, 1988 Fox et al., 1982 Hisanaga & Takeuchi, 1983 Johnson, 1980 Nagymajtenyi et al., 1988 Takeuchi & Hisanaga, 1977 WHO, 1986
Evoked Potentials (EP): General Description	Sensory system is stimulated by physical (e.g., light, sound) or electrical stimulation; integrated electrical response of sensory pathways to the brain and within the brain are measured Knowledge of peak neurogenerators enables localization of damage and helps target neuropathological evaluations	Mercury, carbon disulfide	Most laboratory animals Mice present a technical challenge due to small size	Usually reproducible within and between animals, often between species One EP type gives information about only one sensory pathway (somatosensory, visual or auditory). Implantation of electrodes improves data quality. Parameters of sensory stimulation must be carefully controlled Restraint or anaesthesia of animals is not advisable for cortical or cerebellar EPs Control body temperature when anaesthesia used. Evoked potentials vary with body temperature. Complete equipment not commercially available Special expertise is required that is often not available at contract laboratories Not compatible with standard toxicity studies Satellite groups required	Boyes, 1994 Dyer, 1985 Dyer et al., 1978 Johnson, 1980 Lund & Simonson, 1993 Mattsson & Albee, 1988 Mattsson et al., 1992 Otto et al., 1988 Rebert, 1983 Rebert et al., 1986

Table 5: Tests Which Evaluate the Spontaneous and Evoked Electrical Activity of the Brain (contd.)

Test	Measurement	Agents	Species	Comments	References
Visual Evoked Potentials: Flash evoked potential (FEP) and pattern reversal evoked potential (PREP)	Record integrated responses of visual pathway (from eye to brain) in response to visual stimuli. Flash and pattern stimulation reveal different visual functions	Triethyltin, carbon monoxide	Rats, monkeys, dogs	Abnormalities of the retina and the visual pathway occur in albino animals, limiting their use for visual EP's, although flash-evoked responses may be recorded. Aging albino rodents show high rates of spontaneous retinal lesions. Level of dark adaptation and pupil size may be important. Not compatible with standard toxicity studies. Satellite groups required	Boyes, 1994 Brancack et al., 1990 Dyer, 1985 Dyer and Annau, 1977 Hudnell et al., 1990 Mattsson & Albee, 1988 Otto et al., 1988 Suzuki et al., 1991
Auditory Evoked Potentials (AEP) Brainstem Auditory Evoked Response (BAER)	Record integrated responses of auditory pathway (from ear to brain) in response to auditory stimuli. Potentials generated along the auditory pathway enable localization of damage	Solvents (eg., toluene), styrene, ototoxic aminoglycosides	Rats, monkeys, dogs	Potentials recorded at high stimulus levels may not reflect hearing thresholds due to sensory recruitment. Hearing loss may be frequency-selective, so broad band or limited frequency testing might miss effects. Rodents hear higher frequencies than humans requiring special stimulation equipment. Not compatible with standard toxicity studies. Satellite groups required	Chen & Chen, 1990 Johnson et al., 1988 Legatt et al., 1988 Pryor et al., 1987 Rebert et al., 1982 Rebert et al., 1991 Shapiro, 1994 Simonson & Lund, 1995 Yano et al., 1992
Somatosensory Evoked Potentials (SEPs)	Record integrated responses of somatosensory pathway (from receptors in skin / muscles to brain) in response to mechanical, proproceptive or thermal stimuli. Potentials generated along the somato- sensory pathway enable localization of damage	Lead, carbon disulfide, acrylamide	Rats, monkeys, dogs	Similar equipment as for nerve conduction velocity can be used. Care required to deliver equivalent stimulus levels across subjects. Far-field potentials may be difficult to observe in rodents. Not compatible with standard toxicity studies. Satellite groups required	Allison et al., 1991 Arezzo et al., 1979 Dyer, 1987 Mattsson et al., 1989 Rebert & Becker, 1986 Wiederholt & Iraqui-Mandoz, 1977

Table 6: Tests Which Evaluate Evoked Electrical Responses of Receptors and Peripheral Nerves

Test	Measurement	Agents	Species	Comments	References
Peripheral Nerve (motor and sensory) evoked potential Nerve Conduction Velocity (NCV) (See US EPA Health Effects Test Guideline 870.6850 Peripheral Nerve Function)	Peripheral nerve is stimulated electrically using electrodes, and the conduction velocity of the nerve and the size of the evoked potential of the nerve is measured with electrodes.	Decreased by peripheral neurotoxicants eg., carbon disulfide, hexachlorophene, methylmercury, acrylamide)	Most laboratory animals Mice are challenging due to small size	NCV can be obtained for both sensory and motor fibres independently NCV's reflect only the fastest conducting sensory and motor fibres Measures of size of evoked potential vary with electrodes and electrode placement Requires consistent electrode placement to reduce variability in size of sensory or motor nerve potential Easy to obtain size of evoked muscle response after motor nerve stimulation Animals usually anesthetized, but awake preparations available Important to control temperature since NCV varies with temperature Compatible with standard toxicity studies	Arasaki, 1992 Chen et al., 1992 DeJesus et al., 1978 Glatt et al., 1979 Goto & Peters, 1974 Herr and Boyes, 1995 Maxwell & Le Quesne, 1979 Misumi, 1979 Miyoshi & Goto, 1973 US EPA, 1998a
Electromyography (EMG)	Muscle is stimulated by mechanical stimulation with a small hand-held needle electrode and evoked electrical muscle potentials (insertional activity) are recorded.	Agents affecting neuromuscular function alter discharge patterns (eg., manganese, organophosphates)	Most laboratory animals Mice are challenging due to small size	EMG examination of animals is not as complete as for humans, in which electrical activity during voluntary, graded contraction of muscle can be studied in unanesthetized subjects. Needle electrode insertions may produce histopathological evidence of muscle pathology Compatible with standard toxicity studies	Johnson, 1980 Lukas, 1970 Merletti et al., 1992 Ross & Lawhorn, 1990 Ulrich et al., 1979
Electroretinography (ERG)	Retina is stimulated with light and resulting electrical activity is recorded with an electrode. A-wave reflects photoreceptors; B-wave reflects bipolar and Muller cells	Methanol, sustained bright light	Most laboratory animals Mice are challenging due to small size	Stimulus parameters need to be set precisely. Level of dark adaptation and pupil size are important Animal testing can be based on standards for clinical practice Compatible with standard toxicity studies	Fox & Faber, 1988 Fox et al., 1991 Jones et al., 1994 Nylen et al., 1993 Xu & Karbowski, 1995

Table 7: Tests for Specific Cell Functions

Test	Measurement	Agents	Species	Comments	References
Single Cell Recording	Records spontaneous electrical activity, membrane potential or current from one cell or ion channel	Excitotoxic chemicals (eg., glutamate, pyrethroid insecticides)	Most laboratory animals Usually requires isolated preparation from part of the nervous system (e.g. brain slice, peripheral nerve)	Invasive procedures or require ex *vivo* tissue. Particularly useful for chemicals which can act on pre- or post-synaptic receptors, specific ion channels Can use lower vertebrate modelsSpecial equipment needed that is not usually available at contract laboratories Not compatible with standard toxicity studies although tissues may be removed *post mortem*	Atchison, 1988 Baxter & Byrne, 1991 Kerkut and Heal, 1981 Ravindranath and Pai, 1991 Sakmann and Neher, 1995 Rudy and Anderson, 1992

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