

Animal Behavioral Methods in Neurotoxicity Assessment: SGOMSEC Joint Report

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Introduction

Historically, morphological and classical toxicological methods have been used to provide evidence of neurotoxicity; however, there has been increasing interest, both at the scientific and regulatory level, in the use of animal behavioral methods for evaluating neurotoxicity (1). The increasing interest in the use of behavioral methods in neurotoxicology is based on a number of different factors including *a*) a greater awareness that environmental exposures can produce behavioral and neurological effects; *b*) progress in

basic neuroscience, which has begun to provide greater understanding of the cellular and molecular basis for the behavioral effects of chemicals; and *c*) regulatory activities calling for testing of new and existing chemicals for neurotoxic potential (2).

The use of behavioral end points in toxicology is not entirely without precedent. Cageside observations of neurological and behavioral changes, for example, have been part of toxicological screening studies for many years. However, it has been only recently that the incorporation of more systematic observational methods into a clinical screening battery has been described for the purpose of identifying neurotoxicants (3–5).

In addition to observational methods for the documentation of overt signs of neurotoxicity, a wide variety of behavioral methods and paradigms is also currently available for use in laboratory animals for studying specific neurotoxicant effects. Different behavioral paradigms for measuring sensory functions using conditioned and uncondi-

tioned behaviors, for example, have been successfully used to study visual (6), auditory (7) and somatosensory (8) changes produced by long-term neurotoxic exposures. Behavioral methods have also been developed for quantifying neurotoxicant effects on different aspects of specific motor functions including, for example, neuromuscular strength (9), whole-body and limb tremor (10,11), and alterations in gait (12). Further, cognitive behaviors such as the performance of learned tasks and processes related to attention, learning, and memory are also amenable to study using behavioral methodologies (13). Finally, techniques for studying the effects of chemicals on social and emotional behaviors have also been described (14,15). Behavioral methods for evaluating specific functions and processes require a sound knowledge of the principles of behavioral analysis and often involve the use of automated techniques. Typically such techniques are not found in general toxicology laboratories; however, such methods may be the only practical means of fully characterizing the range of effects of a given compound and may prove invaluable in predicting effects that might be expected to occur in human populations exposed to specific neurotoxicants.

Whether a neurotoxicological study is meant to screen for neurological and behavioral impairments using observational techniques or to evaluate the development of a specific functional deficit using more sophisticated behavioral paradigms, the fundamental aim of testing compounds for neurotoxicity in laboratory animals is to prevent neurological disease in human populations. However, humans are by no means the only species exposed to neurotoxic agents; wildlife populations are also exposed to environmental contaminants. Although studies of the behavioral effects of animals in the wild are scarce, laboratory studies have suggested that adaptive behaviors in different wildlife species may be affected by toxic exposures (16).

Because of the relatively recent appearance of behavioral methods in regulatory activities calling for neurotoxicity testing and the lack of familiarity that most toxicologists have with behavioral principles and methods, a number of concerns have been raised as to the necessity and feasibility of including behavioral end points in neurotoxicity studies. The purpose of the present paper is to discuss the rationale and background for including

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Abbreviations used: PCBs, polychlorinated biphenyls; CNS, central nervous system.

behavioral end points in neurotoxicity assessments, to present the current strategies that have been proposed for identifying neurotoxic agents, to summarize the types of methods and approaches available for characterizing the effects of chemicals on behavior, and to discuss some of the advantages and disadvantages of behavioral methods for studying chemically induced nervous system effects.

Background and Rationale for Behavioral End Points in Neurotoxicity Studies

The Nervous System as a Target for the Effects of Toxic Exposures

Increased industrial activities over the last 100 years have introduced a vast array of new chemicals into the workplace, home, and environment, which has led to a greater risk of chemical exposures both for human as well as for animal populations. With this increase in the number of chemicals in use, accumulating evidence indicates that the nervous system is sensitive to the effects of a number of different chemicals (17).

Much of our knowledge regarding the neurotoxicity of specific chemicals has not originated in the laboratory, but rather has come from outbreaks of human neurotoxicological disease due to environmental and industrial overexposures as a consequence of accident or ignorance. In the occupational setting, a number of outbreaks of neurotoxic disease have occurred as the result of exposure to known and (at that time) unknown neurotoxic agents including pesticides, metals, and organic solvents (18). In addition, outbreaks of chemically induced neurological disease have also occurred in the general population, which emphasizes the fact that the risk of neurotoxicological damage is not confined to the occupational setting. In the United States, for example, several thousand persons were paralyzed during the 1920s as a result of ingesting Jamaica Ginger, a drink containing cresyl phosphates (18). Subsequent incidents involving the contamination of cooking oil with cresyl phosphates in Morocco (19), with polychlorinated biphenyls (PCBs) and furans in the Far East (20), and with unidentified neurotoxic substances in Spain (21) have led to tens of thousands of cases of neurotoxic illness. In addition, contamination of fish with industrial mercury-containing wastes resulted in two large-scale epidemics in Japan, and more than 7000 persons were hospitalized in Iraq as the result of ingestion of grain

contaminated with organomercurial fungicides (22,23). In the incidents with methyl mercury and PCBs, neurotoxicological illness was not restricted to the adult population; children born to exposed mothers also suffered lasting neurological and behavioral effects as well (22,24).

In addition to these dramatic instances of chemically induced neurotoxic disease produced by high levels of exposure, there is also evidence from human epidemiological studies which demonstrates that neurotoxic effects may be occurring in humans at levels considerably lower than those necessary to produce frank manifestations of neurological illness. Epidemiological studies in lead-exposed and PCB-exposed children (25,26) have indicated that developmental exposure to these compounds may produce subtle changes in infant development and cognitive functioning. In addition, cross-sectional studies of workers also indicate an association between exposure to a variety of chemical agents and subclinical changes in neurobehavioral functioning (27-31). Taken together, human outbreaks of neurotoxicological disease and injury and the growing number of epidemiological studies demonstrating the subclinical effects of neurotoxicological exposure in workers and in the general population provide compelling evidence for the potential of different classes of chemicals to affect neurological function and adaptive behaviors in exposed human populations.

In addition to human populations, animal populations both domestic and wild, may also be affected by neurotoxicological exposures. Domestic animals, for example, can potentially serve as sentinels for environmentally mediated neurotoxicological disease or as an additional source of information about diseases occurring in their owner's ambient environment. Domestic pets living in close proximity to their owners share not only living quarters but also, in many cases, their owner's food, water, and exercise habits. One result of this close contact is that pets may share their owner's exposure to neurotoxic substances and the same resulting diseases. For example, one feature of the outbreak of the methyl mercury poisoning associated with Minimata Bay was that domestic cats in households where fish was frequently consumed developed prominent signs of central nervous system (CNS) intoxication (32). Pets may also share their owner's access to modern medicines and develop some of the same untoward effects. Myelin damage associated with hexachlorophene has been

observed both in infants and in puppies bathed with hexachlorophene-containing soaps (33). Further, domesticated farm animals may also be exposed to the same toxicants as their owners and often at higher exposure levels. In this regard, one of the first occurrences of peripheral neuropathy associated with organophosphate pesticide exposure was reported in Leptophos-exposed Egyptian water buffalo (18). In a few instances, zoo animals have also been the source of information about the neurotoxic properties of environmental neurotoxicants. Lead poisoning, for example, has occurred in young primates ingesting paint chips; this has a direct correspondence with lead poisoning in children due to pica (34).

In addition to domestic animal populations, there are also indications that wild animal populations may be affected by neurotoxicological exposures (35,36). Insecticides designed to kill insects by attacking specific sites within the insect nervous system, may produce behavioral changes at sublethal levels both in target and nontarget species. For example, disruption of bee dancing in which the distance and direction of food resources are communicated has been reported following methyl parathion exposure (37), as well as the disruption of foraging behavior for new food supplies following permethrin exposure (38). In field studies conducted in wild populations, experiments carried out in herring gulls employing egg-exchange procedures have provided some indication of behavioral effects (39).

While it is possible that behavioral changes could seriously affect wildlife populations, direct proof of altered behavior in animals in the wild due to the presence of neurotoxicants is difficult to obtain. There are two major difficulties in the testing of wildlife. First, measurements that are most easily quantifiable, such as measures of operant behavior, are not ecologically realistic while behavior such as prey capture is much more difficult to quantify. Second, even if a change can be demonstrated, it is difficult to link it to a specific chemical. For example, despite studies involving thousands of hours of observation (40,41) it was not possible to determine if the suggestion (42) that behavioral changes were involved in the decline of the peregrine falcon (*Falco peregrinus*) was correct. Further, behavioral changes of animals exposed to organophosphorous pesticides have been demonstrated (43,44), but only at 50% or greater inhibition of acetylcholinesterase; this suggests

that it is more practical to rely on the determination of this enzyme in hazard assessment rather than behavioral changes. Although it has been demonstrated that a wide range of behaviors in a wide range of species are affected by environmental pollutants in laboratory experiments, very few behavioral changes have been demonstrated in wild populations. The possibility that behavioral modifications caused by pollutants could result in significant population effects has certainly not been excluded by the studies to date. It is, however, difficult to quantify behavioral changes in the wild, and it is even more difficult to relate these changes unequivocally to population effects and to establish the linkage to a specific chemical or chemicals.

Despite the difficulties inherent in documenting toxicant-induced behavioral changes, there is little doubt that there are significant regional differences in body burdens of neurotoxic pollutants. Thus, it may be possible to monitor body burdens of environmental neurotoxins in wild populations to identify regional differences in contamination (45) or to monitor wildlife population parameters (46) to help predict possible risks to humans and other species.

Behavioral End Points in Neurotoxicity Research

The nervous system is a highly complex organ system that comprises the brain, the spinal cord, and a vast network of peripheral nerves and sensory organs. The nervous system is responsible for receiving, transmitting, and integrating information that allows an animal to react and adapt to its environment. Psychological processes related to behavior such as perception, learning, memory, affect, and voluntary and involuntary movement are all dependent on the adequate functioning of the nervous system. Further, the autonomic nervous system provides extensive innervation of other organ systems involved in homeostatic control of physiological functions such as blood pressure, heart rate, and respiration. Thus, the nervous system exerts executive control over most, if not all, bodily functions.

As a result, nervous system injury can be expressed in a myriad of ways. Some neurotoxicological exposures, for example, can produce frank irreversible neurological and psychiatric disease resulting in coma, convulsions, paralysis, and dementia. However, even slight nervous system damage may impair reasoning ability, cause loss of memory, produce sensory disturbances, interfere

with motor function, and impair health indirectly by reducing functions such as attention and alertness that ensure safety in the performance of daily activities. Table 1 lists some of the different types of effects that have been associated with exposure to toxic chemicals (47).

Toxicant-induced changes in the nervous system can be studied on a number of different levels including electrophysiological, neurochemical, morphological, and behavioral levels. The choice of the most appropriate approach and the methods to be used at a given level of investigation depends on the scientific question under study. It is doubtful that any one of these

Table 1. Human and animal neurobehavioral effects of chemical exposures.

Effect	No. of chemicals producing the effect ^a
Motor	
Activity changes	32
Ataxia	89
Convulsions	183
Incoordination, unsteadiness, clumsiness	62
Paralysis	75
Pupil changes	31
Reflex abnormalities	54
Tremor, twitching	177
Weakness	179
Sensory	
Auditory disorders	37
Equilibrium changes	135
Olfaction disorders	37
Pain	47
Pain disorders	64
Tactile disorders	77
Vision disorders	121
Cognitive	
Confusion	34
Memory problems	33
Speech impairment	28
Affective or personality	
Apathy, languor, lassitude, lethargy, listlessness	30
Delirium	26
Depression	40
Excitability	58
Hallucinations	25
Irritability	39
Restlessness	31
Sleep disturbances	119
General	
Anorexia	158
Autonomic dysfunction	26
Cholinesterase inhibition	64
CNS depression	131
Fatigue	87
Narcosis, stupor	125
Peripheral neuropathy	67

^aThis also includes chemical groups that produce the effect. From the National Research Council (47).

approaches alone can provide a complete picture of a given compound's effects on the nervous system. However, there are a number of advantages in the use of behavioral approaches that make them particularly suitable for studying the effects of toxic exposures on the nervous system.

First, behavior represents the net sensory, motor, and integrative outputs of the central, peripheral, and autonomic nervous systems and, as such, can be used to provide an index of chemically induced changes in nervous system function. In addition, behavioral methods are noninvasive and can be used to measure acute effects and to track the progressive development of neurotoxicity during long-term exposure in chronic studies. Further, the first signs of neurotoxicological effects in humans are neurological and behavioral in nature, and animal models that can predict these early effects are of obvious importance.

In addition, there are a number of other qualitative features of neurotoxicity that have profound consequences for evaluating and predicting neurotoxic risks outside the laboratory (48). Neurotoxicological effects may be cumulative and progressive, and multiple functions are often affected as the degree of exposure is increased. Neurotoxins can produce silent and covert damage that may not be readily apparent unless the adaptive capabilities of the organism are challenged in some way. The expression of neurotoxicity may be age related such that the effects of the normal aging process are compounded with deficits from previous neurotoxic exposures. A consideration of these aspects of neurotoxic effects is particularly important for the characterization of neurotoxicological risks. Because such effects are typically expressed on a functional level, behavioral approaches are the most logical and economical means available for addressing such problems.

Behavioral Approaches

Historically, different schools of psychology have contributed to the development of the scientific study of behavior and, in turn, to present day approaches in behavioral methodologies. Ethology, for example, places a heavy emphasis on observational methods for studying naturalistic behaviors and was developed to a great extent through the work of European scientists (49). In North America, the study of behavior has been more focused on elucidating the principles underlying conditioned or learned behavior.

Ethology provides a variety of observational tools to study behavior using less rigidly controlled environments than those typically employed in traditional experimental psychology. Methodologically, this approach makes use of ethograms, which consist of a detailed series of carefully defined behavioral responses representing different categories of the behavioral processes of interest (50,51). Ethological approaches are particularly indicated in studies aimed at characterizing alterations in social behavior (52). By focusing on behaviors occurring under seminatural conditions, ethologically oriented laboratory investigations permit the assessment of a wide range of behaviors, which can allow for a sophisticated analysis of behavioral abnormalities as a result of chemical exposure or other experimental manipulations (45).

In contrast, experimental psychologists have historically emphasized investigations aimed at establishing general principles that underlie learning and memory processes. By concentrating on the rigorous control of variables that govern behavior in experimental laboratory studies, experimental psychologists developed a broad range of behavioral paradigms to investigate the proximate causes of behavior (54), i.e., the combination of exogenous and endogenous variables which produce a particular behavioral outcome at a particular point in time. The sophistication of these techniques permits the conduct of tightly controlled behavioral studies that can be used to characterize the effects of drugs, chemicals, and other experimental manipulations on memory, learning, sensory, and motor processes.

Despite the methodological and theoretical differences among different approaches in psychology, behavioral scientists conceptualize behavior as identifiable units termed responses or as patterns of responses that occur in a spatial and temporal framework. Further, operational definitions are used to define different psychological processes in terms of these responses. The operational definition of psychological processes in terms of the occurrence of specified identifiable responses is extremely important since it allows for the analysis of the occurrence, frequency, and patterning of different behaviors.

Strategies for the Use of Behavioral Methods in Toxicology

Given the evidence that chemical exposures may have serious health effects in human and animal populations, it is somewhat surprising that animal testing to examine

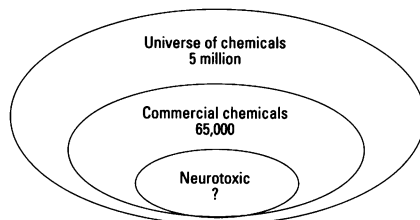


Figure 1. Out of the 65,000 chemicals presently on the market, the number of chemicals that are neurotoxic is unknown.

the neurotoxic effects of chemical exposures has not been initiated on a wider scale. However, as depicted in Figure 1, for most of the 65,000 chemicals currently in commerce as well as the 2,000 new chemicals that are introduced on to the market each year, relatively few have been tested for neurotoxicity (55). Even for compounds such as pesticides, which in many cases are designed to act through toxic effects on the nervous system of lower animals, less than 10% have been sufficiently examined to determine their possible consequences to the nervous system. Although it is impossible to state with certainty the number of neurotoxic compounds already in existence, reviews of the literature and current databases (55,56) estimate that 3 to 28% of all chemicals in the environment possess some neurotoxic activity.

In response to this lack of information, there has been an increase in regulatory activity aimed at developing strategies for testing for neurotoxicity at the animal level, which includes behavioral end points (57–60). The approach most frequently put forth for the regulatory testing of neurotoxicity is a tiered testing approach (1,47,61), although there is still debate as to which behavioral tests should be included in which tier. The purpose of testing chemicals at the Tier 1 level would be to identify chemicals with neurotoxic potential. Because it is envisioned that behavioral tests would be included in the routine testing of chemicals for regulatory purposes, methods that are simple and economical to perform and require no pretraining of the animals are typically the techniques which have received the most attention. The most common behavioral tests being proposed consist of standardized observations using operationally defined end points, several manipulative tests to assess several aspects of sensory/motor functions, and the automated assessment of motor activity. In the logical scheme of such a tiered testing strategy, a compound identified at the Tier 1

level would subsequently undergo further testing (Tier 2) using more advanced behavioral techniques to better characterize the neurotoxic effects of the compound and to determine dose–response relationships for risk assessment purposes. Behavioral tests conducted at the Tier 2 level would be aimed at objectively quantifying sensory and motor deficits as well as evaluating cognitive behaviors related to learning, memory and performance.

An overview of the behavioral techniques currently being proposed for inclusion for neurotoxicity screening are discussed below. In addition, a number of techniques suitable for characterizing different types of behavioral impairments are also presented.

Behavioral Neurotoxicity Screening Techniques

Observational Methods for Documenting Clinical Signs

Observation is a part of every scientific discipline, and the scientific study of behavior is no exception. Behavioral observations can provide information regarding the appearance of both overt neurological abnormalities such as convulsions, paresis, and ataxia, as well as behavioral abnormalities characterized by changes in an animal's responsiveness to its environment. Direct observation of an animal's behavior following exposure to a chemical agent is one of the most straightforward means of documenting clinical signs of toxicant-induced neurological and behavioral impairment and is a logical starting point for investigating the potential neurotoxic effects of a compound for which neurotoxicity data are lacking.

For observational methods to be effective, a structured protocol covering different functional domains should be used and applied in a systematic fashion. Since observational methods are used in the early stages of hazard identification, it is important that different aspects of nervous system function be included in the examination. The methods must cast a broad net to catch neurotoxicants that potentially can have many different effects on neural functioning. Typically, most observational screening batteries currently in use include items designed to provide information on the presence and severity of convulsions, tremor, gait disturbances and other motor abnormalities, the functioning of different stimulus modalities, autonomic function, and general reactivity (4,62). To increase

Table 2. End points included in a functional observational battery in the WHO/IPCS collaborative study on neurotoxicity assessment.

Home cage and open field	Manipulative	Physiologic
Posture	Ease of removal	Body temperature
Convulsions and tremors	Ease of handling	Body weight
Palpebral closure	Palpebral closure	
Lacrimation	Approach response	
Piloerection	Click response	
Salivation	Tail-pinch response	
Vocalizations	Righting reflex	
Rearing	Landing foot splay	
Urination	Forelimb grip strength	
Defecation	Hindlimb grip strength	
Gait	Pupil response	
Arousal		
Mobility		
Stereotypy		
Bizarre behavior		

Data from Moser (4).

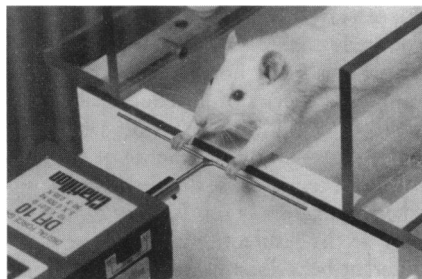


Figure 2. Neuromuscular weakness can be assessed using commercially available strain gauges to measure forelimb and hindlimb grip strength. (Photo credit: BM Kulig).

the sensitivity of observational screening methods, the highest dose groups in neurotoxicity studies typically are set at minimally toxic or limiting-dose levels. Several protocols for use in neurotoxicity assessment have been described (3–5). An example of items usually included in a protocol for a functional observational screening battery is presented in Table 2. Such a battery typically comprises direct measurement in the home cage and in an open field, as well as several manipulative tests to evaluate sensory reactivity and motor function. One semiquantitative test that has proved particularly robust and is employed in a number of laboratories' commercially available strain gauges to obtain estimates of forelimb and hindlimb grip strength (Figure 2).

Although observational methods are perhaps conceptually the most straightforward, they are also the easiest to confound and can sometimes be difficult to interpret unless there is some internal or external corroboration of results. For example, a correspondence between ease of removal from the home cage and reactivity to being

handled provides more convincing evidence of a functional deficit than an effect on one measure alone. For this reason, some investigators have explored the use of composite scores designed to reflect functional integrity within a given domain (63). Likewise, external corroboration in the form of data obtained with another test system is also helpful, e.g., a reduced response to an auditory stimulus may indicate a sensory hearing loss. However, it could also be a function of motor changes or some aspecific effect related to arousal. Data from experiments using electrophysiological methods for measuring auditory evoked potentials, behavioral experiments designed to measure auditory thresholds, or histological evidence of damage to the cochlea would be necessary to determine the specificity of effects on the auditory system per se.

Because scientists in general have quite a bit of experience in making observations, it is often assumed that this general observational experience provides a basis for expertise in making behavioral observations. Without a knowledge of neurological functions and the normal behavior of the species under study, untrained observers of behavior are likely to make observations in a highly selective manner, to miss effects of importance, or to fail to carry out these methods in a reliable fashion.

Because of the subjective nature of the observational screening battery, the use of a structured neurological/behavioral examination under standardized conditions and using observations with clear, operationally defined end points are recommended. Not only does a structured protocol reduce personal bias, it also can help in training observers. Further, to ensure as far as

possible that observer bias is minimized or controlled, observational methods should also be carried out in a blind fashion, with the observer unaware of the treatment of the animal.

Observational methods for screening purposes can be used both in adult and developing animals. Although similar observational end points are used for studying behavior in animals of different ages, there are some important differences in the interpretation of these similar, but different, assessments. In fact, in immature rats and mice, the time of first appearance and subsequent maturation of several reflexes and responses shows remarkable regularity. This means that not only abnormal responses but also changes in the time of appearance and maturation of otherwise normal responses can be exploited in the assessment of effects in developing organisms (52,64).

Although a number of papers have been published on the use of observational methods for neurotoxicity screening, not all authors have reported equal success in detecting the effects of neurotoxicants with this approach (65). Thus, interlaboratory data are necessary regarding the sensitivity of observational methods in detecting neurotoxicants as well as information regarding interlaboratory and intralaboratory reliability. In this regard, the International Programme on Chemical Safety of the World Health Organization is currently sponsoring an international collaborative study on neurobehavioral methods for neurotoxicity screening in which eight laboratories are participating (66).

Motor Activity Assessment

Similar to observational methods, motor activity assessment requires no prior learning on the part of the animal and thus may be a useful method for neurotoxicity screening. Because the technique has been used extensively in behavioral pharmacology for many years, quite a bit is known about the sensitivity of activity measurements to the effects of different drugs and brain damage, as well as the advantages and limitations of different measurement devices (67–69).

Different approaches to detecting the movement of animals include field detectors, activity wheels, photocell-based systems, and video-based devices. Although all of these systems detect movement, there are very large differences in the type of motor activity that they measure. One example of a field detection device, for example, is based on the generation of a

capacitive field around the test chamber. In this system, an adjustable oscillator supplies high-frequency current to an input coil, which creates a field around the test chamber. Movement within the test chamber produces momentary changes in the voltage in the output coil, which is digitized and reported as an activity count. One of the disadvantages of field systems is that any movement large enough to activate the coil may be detected. This means that movements other than spontaneous locomotion, such as body-part movements, grooming, and even tremors and convulsive movements will be included in the measurement, making the interpretation of effects on locomotor activity per se difficult.

In contrast to field detection devices, activity wheels tend to measure a very specific type of ambulation under very specific circumstances. Activity wheels are electromechanical-based devices that consist of an enclosed wheel attached to the animal's home cage. When the animal enters the wheel, it tends to run; the measure of activity is the number of revolutions that the animal makes in the wheel. All other activities, including eating, drinking, grooming, exploring, etc., occur in the home cage portion of the apparatus. The major drawback of activity wheels is that the movement itself provides feedback to the rat which can modify running rate in a rather unspecified manner. This is perhaps one of the reasons underlying the large differences between rats in this type of activity measurement.

To obtain a more refined measure of horizontal locomotor activity in a stationary environment, detection methods employing either photocells or computerized video-imaging techniques are typically employed. In photocell-based systems, the photocells are positioned in such a way that they are primarily sensitive to horizontally directed movement which is measured in either simple test environments, such as a rectangular box or circular enclosure, or in complex maze-type environments, such as the figure-8 maze or the residential maze.

In video-based systems, a camera positioned above the test apparatus receives a video image of the white rat on a black background, which is digitized by computer into a series of X-Y coordinates; this provides information on the amount of ambulatory movement in terms of total meters run, the distribution of movement at different speeds, and the amount of locomotor activity in different locations within the test chamber.

Not only do motor activity assessment methods differ with respect to the type of detection device used; there are also many different types of testing conditions that can affect motor activity assessment: familiarity of the animal with the test situation, illumination conditions, complexity of the test environment, the length and frequency of the test period, and the age of the animal. These factors must also be taken into account when designing either screening protocols or experiments to more fully assess activity changes (67-69).

In neurotoxicity testing for regulatory purposes, motor activity is typically measured outside the home cage using either a simple or complex environment. Typically, a test period of 30 to 60 min is used based on the rate of habituation engendered in a specific device (70).

Issues raised over motor activity assessment for the purposes of regulator neurotoxicity screening fall into two categories: technical and conceptual (70). Objections raised on a technical level include discussions of the variability of motor activity data, the reliability both within and across laboratories, and the sensitivity with which effects can be detected. On the conceptual level, concerns usually involve the specificity of changes in activity, particularly for indicating the mechanisms underlying the degree and direction of activity changes (71,72).

With respect to technical concerns such as reliability, etc., data available thus far from the many hundreds of studies of psychopharmacological agents, as well as different types of chemicals, provide quite compelling evidence that motor activity assessment is a sensitive and valid means of measuring chemically induced changes in activity. Further, in a retrospective study comparing data from six different laboratories, similar results were found on this measure despite variations in the conditions under which the studies were conducted (73).

With respect to conceptual issues regarding specificity, it has been argued that motor activity assessment constitutes an apical test, i.e., one which requires the integration of a variety of systems and thus might be particularly useful for screening purposes (68). Of course, it is important to distinguish chemicals' effects on neural function from effects on other systems. However, from the data thus far available, it does not appear that decreases in motor activity simply reflect malaise or general illness (74).

Tests for Specific Behavioral Functions

Although observational methods and motor activity assessment constitute a rational approach to first tier screening, there are a number of important psychological functions such as memory, learning, attention, and social behavior that are beyond the scope of these methods. Further, a detailed characterization of specific sensory deficits is likewise unfeasible with this approach. To evaluate these and other effects, there must be methods that go beyond the documentation of the occurrence of clinical and behavioral abnormalities at high-dose levels.

An improved characterization of a behavioral deficit is typically achieved through the use of more complex tests specifically designed to assess and differentiate sensory, motor, or learning/memory and performance functions. In this context it should also be noted that each of these various categories of function is also composed of component behaviors which may be separately evaluated. Some of the more advanced tests permit the determination of whether changes in one behavioral function may be the indirect result of changes in a different behavioral function. For example, changes in learning may be an indirect consequence of an impaired ability to detect or respond to the environmental stimuli that are critical to the task. It may be necessary to evaluate behavior on several different complex tasks to fully determine the profile of behavioral toxicity.

Tests of Motor Function

Exposure to many different classes of substances (including metals, solvents, pesticides, gases, and drugs) has been associated with toxicant-induced motor disturbances. In some cases, functional effects are the result of damage to peripheral nerve while, in others, structures within the central nervous system are involved. Not surprisingly, the types of motor effects that have been reported in the literature for different compounds are equally heterogeneous, ranging from specific deficits such as ataxia, paresis, tremor, and other types of dyskinesias to subtle changes in the force and duration of specific motor acts.

A number of techniques have been developed for measuring the effects of different aspects of motor function in small laboratory animals and have been reviewed in recent papers (69,75,76). These tests differ widely with respect to the types of motor deficits that they are designed to measure and the degree to which they are

Table 3. Tests used to evaluate effects on motor function.

Effect	Behavioral test/end point	References
Neuromuscular weakness	Grip strength	(9,77–80)
Endurance	Swimming test	(81,82)
Ataxic and paretic gait	Gait analysis	(83–85)
Incoordination	Negative geotaxis	(86)
	Rotorod	(87–90)
	Treadmill	(91)
	Coordinated movement test	(12)
Impaired motor execution	Force and duration	(82–94)
Tremor	Tremor quantification	(10,94)
Catalepsy	Bar test	(95)
Stereotypies	Observation	(96–98)

automated [Table 3; (77–98)]. There are considerable differences in the psychometric properties of different tests of motor function and their suitability for application in long-term experiments.

Some of these paradigms are relatively simple approaches that do not require extensive training or pretesting of the animal; however, some of these tests are also susceptible to confounding. Rotarod testing, for example, has been widely used in acute pharmacological experiments to provide dose-response data on the effects of drugs on motor coordination. With repeated testing, control animals tend to jump off the rotating rod, which makes interpretation of results impossible (69,75). Other simple tests such as gait analysis are quite labor intensive and may be influenced by the speed of ambulation as well as the size of the animal (84,88).

Some of the more advanced techniques offer more selective assessment of motor function, e.g., computerized tests of motor coordination that use a computerized video-based recording system to analyze the temporal and qualitative characteristics of hindlimb movement under standardized controlled conditions (12). Further, operant techniques also permit the quantification of variables such as force and duration of selected limb movements and the detection and analysis of tremor (11,76,92,93). Such techniques demonstrate the high degree of selectivity that can be achieved using behavioral methods for measuring motor impairment.

Sensory Function

Integrated human function relies on intact sensory capabilities, any or all of which may be affected by neurotoxicant exposure. Visual system deficits have been reported in response to exposure to metals such as methylmercury (6). Auditory deficits are associated with exposure to

aminoglycoside antibiotics, trimethyl tin, and some organic solvents (100).

As with motor function, the techniques available for evaluating sensory function range from relatively simple techniques, such as the tests of sensory reactivity included in most observational batteries, to more advanced techniques. Specific paradigms for studying toxic effects on the visual, auditory, and somatosensory systems have been discussed in several recent reviews (7,101,102). Some sensory functions, e.g., olfaction and taste, have received little experimental attention as yet in neurobehavioral toxicology, although the same techniques could generally be applicable to assessments of these modalities as well.

Basically two different types of behavioral paradigms have been described for evaluating the effects of chemicals, those based on operant conditioning and those using reflex modification techniques. Instrumental or operant conditioning techniques have included the use of both active avoidance paradigms, as well as psychophysical operant discrimination methodologies, in both rodents and primates.

One behavioral technique using avoidance learning to evaluate sensory function is the multisensory conditioned-avoidance paradigm. In this test, animals are first trained on an avoidance conditioning task with different sensory cues such as light, tones of different frequencies, and mild shock as the conditioned stimuli. Sensory impairments, for example—auditory threshold changes—are apparent when animals fail to make avoidance responses to tones while continuing to make responses to stimuli in other modalities. Using a multisensory conditioned-avoidance paradigm such as this, investigators have recently uncovered a neurotoxicological effect of organic solvents not previously measured in laboratory animals with other methods, namely the ability of some

organic solvents to produce irreversible hearing loss (100).

Psychophysical operant discrimination techniques provide a very elegant approach to the evaluation of neurotoxicant-induced specific sensory deficits. In this technique, animals are first trained to emit a specified response in the presence or absence of a stimulus of a particular modality (101,102), i.e., the animal is rewarded for reporting whether it can detect a stimulus by making a particular response. To study the effects of acrylamide on vibratory sensation, for example, Maurissen and his co-workers (8) trained monkeys to hold down a response lever with one hand and to let go of the lever if a vibratory stimulus was detected to the fingertip of the other hand. Each trial was signaled by a tone, and the animal was rewarded if it released the lever when the vibratory stimulus was delivered. To control for guessing, trials in which no vibratory stimulus was delivered were also presented during the test session, the monkey was also rewarded if it did not release the lever during the trials where no stimulus was presented. Similar methodologies have also been adapted to study the effects of acrylamide on the visual system (103) as well as the sensory deficits produced by developmental methylmercury exposure (6).

Although psychophysical techniques constitute one of the most precise approaches to the study of sensory deficits, relatively long periods of time are required to achieve stable baseline levels of responding. Thus, the technique is unsuitable for testing large numbers of animals.

A relatively recent addition to the behavioral methods for auditory assessment is the reflex modification paradigm (7). The technique makes use of the fact that a brief low-intensity stimulus will attenuate the magnitude of the startle response elicited by a subsequent high-intensity stimulus. The technique requires no prior training of the animal, and relatively extensive audiometric testing can be accomplished within a matter of days instead of weeks. Thus, this approach holds a considerable amount of promise for evaluating specific sensory deficits.

Cognitive Behaviors

Behavioral impairments indicative of cognitive changes have been associated with exposure to a number of chemicals. Developmental exposure to lead, methylmercury, and PCBs have been causally related to delayed development and intellectual impairments in children. In adults,

chronic exposure to organic solvents has been associated with the development of toxic encephalopathy characterized by memory loss and cognitive impairments. Given the fact that intellectual abilities related to memory and learning capacity are of such importance in successfully adapting to changes in the environment, it is not surprising that concern has been raised regarding the need to include measures of learning and cognition in evaluating the health effects of drugs and chemicals.

Learning is not a unitary phenomenon and, as a result, many models have evolved to evaluate different aspects of learning and other higher order functions in animals. Some studies have concentrated on the effects of chemical exposures on the performance of learned behaviors, using, for example, free operant or discrete trial techniques; others have attempted to develop models to study acquisition and memory. A variety of different types of testing environments such as two-compartment shuttle boxes, mazes, and operant chambers have been used with different behavioral paradigms including avoidance learning, reversal learning, repeated acquisition, and delay tasks (13,75–99). Although the list is far from exhaustive, Table 4 summarizes some of the more frequently used techniques to study the effects of chemicals on different aspects of cognitive behavior.

As with other behavioral tests, the advantages of the simple approaches are that little training of the subject is required and equipment costs are minimal. However, they often suffer one major disadvantage: it is quite difficult to determine whether observed deficits actually represent changes in cognitive function or are secondary to other functional impairments such as sensory, motor, or activity changes.

One example of such difficulties is encountered in the use of mazes to study learning. The water maze is currently used somewhat extensively to study learning and memory. In this paradigm, a rat is placed in tub of water that has been made opaque through the addition of a substance such as milk powder. The animal's task is to find a hidden platform submerged just below the surface in order to escape from the water. Learning is indicated by a decrease in the latency to find the platform across trials. There are, of course, several ways in which latency measures could be lengthened in such a procedure, suggesting learning impairments without any real change in cognitive function. For example, rodents are known to use visual cues in the surrounding environment in maze situations. Thus, visual deficits could contribute to changes in latency independently of learning. Moreover, changes in motor function might make the swimming response more effortful or less coordinated, thereby also increasing latency independently of changes in cognitive function. Similar confounding influences in the form of changes in spontaneous activity have also been described for passive avoidance learning techniques (13).

More advanced procedures for assessing cognitive functions, such as the multiple repeated acquisition and performance paradigms, can be specifically designed to address such problems (13,75). In this paradigm, animals are required to learn a sequence of responses during the repeated acquisition component of the session, and they only have to execute a sequence of responses that has already been learned during the performance component. Both the repeated acquisition and performance components require animals to have intact sensory capabilities, adequate motor functions and sufficient motivation to perform

the sequence of responses. However, learning per se is only required in the repeated acquisition component of the task. Thus if a toxicant induces selective or direct effects on learning processes, changes in accuracy would be detected in the repeated acquisition only. In contrast, if the toxicant produces changes in sensory, motor, or motivational processes, these effects would be manifest in both the repeated acquisition and performance components of the task.

In addition to memory and learning processes, performance of learned behavior as a result of chemical exposure has also been frequently studied. Different paradigms include both discrete-trial techniques and schedule-controlled behavior using intermittent schedules of reinforcement (99). Put simply, a schedule of reinforcement defines the rules governing the relationship between an operant response and its reinforcing consequences. Different schedules of reinforcement generate marked differences in the pattern and frequency of behavior in time, which are quite characteristic for any particular schedule and show extensive species generality. Schedule-controlled behavior has been extensively used in behavioral pharmacology and is being increasingly applied to the study of toxicant effects (104). Ease of automation, well-studied paradigms, a large database of drug and chemical effects, commercial availability of standard equipment, and a high degree of reproducibility and sensitivity are some of the features that argue in favor of this approach to neurotoxicity evaluation. Thus, schedule-controlled operant behavior offers a standardized but flexible approach for investigating neurobehavioral toxicity.

Social Behaviors

The investigation of social behavior in animals presents several concerns that are not usually present when studying individual subjects. Social behaviors are never unitary events. They are multimodel in nature and consist of exceedingly complex interactions between each participant, their individual physiological and cognitive state, and the environment in which the behavior(s) occur. The many facets of even the most simple social interaction make automated data collection impractical at best and usually necessitate the use of observational (i.e., ethological) methodologies. The particular requirements associated with such techniques have been reviewed by a number of authors (36,51).

Table 4. Conditioning paradigms used to study the effects of neurotoxicants on cognitive behaviors.

Testing environment	Behavioral paradigm	Cognitive function
Two-compartment test chamber	Passive avoidance learning Shuttle box avoidance learning	Short-term memory Learning acquisition, memory, performance
Mazes		
T-maze	Learning Delayed alternation Discrimination reversal	Acquisition Short-term memory Learning
8-Arm radial maze	Spatial memory testing	Short-term memory
Morris water maze	Spatial learning	Short-term memory, learning
Operant chamber	Intermittent schedules of reinforcement Discrete-trial operant discrimination Delayed alternation Discrimination reversal Repeated acquisition	Learned performance Learned performance Short-term memory Learning Learning

Because observational techniques all require judgments to be made by the observer, it is most important to provide narrowly defined operational definitions for the behaviors of interest. Social interactions in rodents have been evaluated for a number of compounds, and provide a ready example. Adult rodent social behavior comprises four primary categories: flight or submissive behaviors, sexual behaviors, aggressive behaviors, and investigative or exploratory behaviors (105). It should be clear that each of these broad behavioral categories consists of many specific behaviors. In evaluating a number of neurotoxicants, Silverman (105) selected several different specific behaviors within each category, provided discrete operational definitions for each, and recorded the rate of occurrence following introductions or reintroductions of one rat to another. There are several characteristics of ethological analyses of social behavior that make this approach well suited for behavioral toxicology. First, the evaluation of social behavior using unobtrusive observational techniques minimizes more stressful experimental manipulations. Second, it places an emphasis on obtaining effects at the lowest end of the dose-response curve. Lastly, as the animals are engaging in natural behaviors rather than ones shaped by the investigator, results may be more readily extrapolated to exposures and effects that may occur outside of the laboratory.

Questions in developmental neurotoxicology can likewise be addressed through the study of social behavior. Interactions between mother and young provide a rich variety of behavior. Typically, analyses of maternal behavior in rodents include ratings of nest constructions, time required to retrieve pups displaced to the far side of the home cage, percentage of observation time the mother spends grooming and nursing the pups, and the amount of exploratory behavior undertaken by the offspring. Barrett and Livesey (106) used these techniques to assess the effects of chronic lead

exposure and found that lead-induced maturational delays in pup development were reflected by increased nursing and decreased exploratory behavior. In addition, play behavior may also be useful in understanding the developmental effects of specific compounds. In a study by Holloway and Thor (107), for example, a number of operationally defined behaviors associated with play-fighting and exploration were found to be increased following developmental lead exposure while no significant alterations in other social activities such as maternal behavior were seen.

Concluding Remarks and Recommendations

There is a large number of behavioral methods available for screening and characterizing neurotoxic effects. From the database presently available, standardized observational methods and motor activity assessment would appear to be appropriate for the initial screening for neurotoxicity. These techniques are technically simple to implement and are thus potentially useful in situations in which resources are limited to address neurotoxicity concerns. Results from ongoing studies such as the WHO/IPCS collaborative study, as well as those from individual laboratories, will help address concerns regarding issues of reliability, replicability, and sensitivity of these methods. However, the prevention of outbreaks of neurotoxicological disease that have occurred in the past through the implementation of these methods will be the ultimate criterion for judging their utility as screening methods.

In light of the recent emphasis on screening, it is possible to lose sight of the fact that sophisticated behavioral paradigms have been developed for measuring specific behaviors under a variety of laboratory and natural conditions. The potential importance of these methods in characterizing and understanding neurotoxic effects has not been fully explored. Thus, the laboratory scientist interested in studying the

range of effects produced in human and animal populations should not be deterred simply because such paradigms are not the focus of regulatory toxicology.

Based on these considerations and the discussion above, the following recommendations for future developments in the area of behavioral neurotoxicology are outlined below.

- Animal behavioral data, whether experimental or ethological, should be used proactively and reactively to address neurotoxicology problems
- Many neurotoxicology studies using laboratory animals are conducted at high-dose levels; more attention needs to be directed at conducting studies at lower environmentally relevant exposure levels
- Simple, sensitive, cost-effective tests for learning and memory should be developed for incorporation into the early stages of neurotoxicity testing
- Tests for specific sensory impairment should be considered for further development and possible inclusion in neurotoxicity testing
- The value of reactive studies, which has been adequately proven in the case of agents or doses with marked harmful effects, should be further verified with agents or doses that have a lower toxicity profile but are still a cause of concern because of the borderline effects they might produce in a large number of people
- Many laboratory studies indicate that wild animals can be affected by neurotoxic chemicals; neurotoxicity field studies on wild animal populations, which are largely unavailable, should be conducted
- When behavioral data are to be used in ecotoxicological risk assessment, techniques to correlate behavioral effects with biomarkers of exposure e.g., residue levels in wild populations or other biomarkers of effect such as cholinesterase inhibition should be developed.

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