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NEUROBEHAVIORAL TECHNIQUES TO ASSESS THE EFFECTS OF CHEMICALS ON THE NERVOUS SYSTEM[1]

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INTRODUCTION

Rationale for the Study of Behavior

Of the many organ systems potentially affected by hazardous agents, the nervous system is one of the most complex and probably the least understood. All bodily functions depend on processing sensory input and generating control output via the nervous system. The complexity of the interaction of the nervous system with other organ systems suggests that nervous system function should be among the first and most thoroughly assessed in cases of exposure to hazardous agents. However, the intricacy of the nervous system makes assessment difficult in at least two ways. First, there are many different functions that could be assessed, ranging from sensory alterations to motor deficits to associative dysfunction. Second, there are no or few guidelines for correlating most neurobehavioral functional deficits with specific neuropathological or neurochemical changes. The purpose of this review is to develop a rationale for the use and selection of behavioral tests to assess agents for potential neurotoxicity.

A cursory review of the current literature indicates that functional measures of central nervous system (CNS) integrity, in particular behavioral techniques,

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are being used with increasing frequency to determine the potential neurotoxicity of environmental agents. Mello (1) was among the first to argue that the behavior of the organism represents a functional integration of the nervous system and that nervous system capacity cannot be determined in histological or even physiological studies independent of behavioral analyses. Many researchers have subsequently pointed out the potential sensitivity of neurofunctional measures to study the deleterious effects of environmental agents (2–5).

In the past, the United States, Canada, and Western Europe placed heavy emphasis in chemical toxicity studies on defining pathological changes following exposure. However, in 1961, Elkins (6) published a paper comparing the maximum acceptable concentrations (MACs) of certain substances in the US with those in the Soviet Union. The higher values adopted by the US were believed to be due to the use of morphometric measures by that country (lethality, body weight loss), while the Soviets relied on functional measures (conditioned reflexes). Subsequent publications by Ruffin (7) and Brimblecombe (8) urged the use of behavioral measures in Western countries to establish MACs.

It is also interesting to note that Citovic (9), a student of Pavlov, reported in 1930 the use of conditioned reflexes to study the neurotoxic effects of gasoline and acetone. In the US, it was 1969 before Weiss & Laties (10) included a section on behavioral toxicology in their chapter on behavioral pharmacology in the *Annual Review of Pharmacology*. Since that time, behavioral toxicology has been the subject of numerous books, symposia, reviews, and book chapters in Western countries (1, 3–5, 11–20).

Scope of the Problem

In the US, the Chemical Abstracts Service Computer Registry contained over four million distinct entities as of 1977, and as many as 100,000 chemicals may be in everyday use by American industry. According to the National Institute for Occupational Safety and Health (NIOSH), more than 20 million people work with one or more chemicals known to be neurotoxic.

Although many of the chemicals that already exist in the environment and the approximately 1000 new chemicals introduced each year are probably not neurotoxic, we will inevitably be exposed to some that are. Many industrial chemicals will enter the environment as waste products discharged into the air, rivers, and lakes, while some will eventually appear as contaminants in food, water, and air. A few will also find their way into the food chain through carelessness or ignorance.

Obviously, an extensive assessment of all the possible chemical entities for potential behavioral and neurological toxicology is not a viable economic option. There is a need for a well-conceived screening program that would provide an initial indication of a chemical's potential to produce neurotoxicity.

The results of the screen could be used to prevent an agent from being employed inappropriately in the marketplace or to suggest additional studies that might provide a more detailed examination of the agent's effects.

Examples of Neurotoxicants

Before discussing specific criteria for the development of a screen for neurobehavioral toxicity, several general factors that may affect test results should be considered. Among these are choices of sex, age, species, and dosing parameters; they have been discussed in detail elsewhere (21–23). Another general consideration concerns the range of possible effects likely to be encountered when attempting to assess neurotoxic chemicals. The following sections illustrate this point by briefly providing examples of signs and symptoms reported in humans exposed to representative neurotoxicants and comparing these effects to those reported in animals exposed chronically to the same neurotoxicants. In the examples below, the rat is used as the animal model, since this or some other rodent species is likely to be used for screening.

INORGANIC LEAD The neurotoxicity of inorganic lead has been known for many years. Encephalopathy has been reported following lead intoxication, as have tremors, disorientation, neuropathy, aphasia, blindness, convulsions, and coma (24). The neuropathy is primarily motor and histologically shows primary changes in the Schwann cells, with segmental disintegration beginning at the nodes of Ranvier (25, 26).

In spite of the well-documented effects of inorganic lead in adult humans, there have been relatively few studies on the effects of low-level exposure to lead in adult animals. High doses of systemically administered lead have been reported to produce motor impairment in a water maze (27) and alterations in the performance of schedule-controlled behavior (28). Lanthorn & Isaacson (29) reported reduced rates of spontaneous alteration in a maze as well as altered reactivity to handling. Pryor et al (30) used a battery of neurobehavioral tests with rats chronically exposed to lead acetate and found significant decreases in spontaneous motor activity (SMA) associated with decreases in body weight. Changes in responsiveness to noxious or nonnoxious stimuli were not detected by the battery of tests used by Pryor et al (30).

SOLVENTS The neurotoxicity of the organic solvents has also been well-documented in humans. Neurological symptoms that follow exposure to these agents include paresthesia, reflex attenuation, symmetrical distal ascending motor weakness, and unsteady gait (31–33).

In rodents, several studies have been reported on the neurotoxic effects of acrylamide (30), which in many cases seems to be prototypic for this class of neurotoxicants. Using relatively simple measures of neuromotor function,

repeated exposure to acrylamide has been reported to decrease forelimb grip strength and depress the hindlimb extensor reflex (34). Pryor et al (30) also reported that acrylamide decreased fore- and hindlimb grip strength, with impairment of the latter preceding alterations in the former. With prolonged exposure to acrylamide, more severe motor debilitation is seen and is quantified as disruption of motor coordination (negative geotaxis) and decreased motor activity (30, 34). Changes in responsiveness to noxious and nonnoxious stimuli have been observed but could not be demonstrated in the absence of motor weakness. The observation that acrylamide primarily affects neuromotor function has also been made by several investigators who have found acrylamide-induced hindlimb splay (35), altered gait (36), and impaired rotorod performance (37).

ORGANOCHLORINE INSECTICIDES Included in the class of organochlorine insecticides are compounds with such widely divergent structures as DDT, chlordane, dieldrin, and chlordecone. Generally, these agents produce tremor, hyperexcitability, and convulsions at higher doses. Chlordecone is a polycyclic ketone formerly used to control fire ants and other insects. Workers exposed to large quantities of chlordecone exhibited a well-defined tremor associated with an ocular flutter (opsoclonus). In addition, exposed workers reported irritability and mild impairment of short-term memory (38). Taylor (39) has subsequently reported that virtually all of the effects produced by chlordecone are reversible.

There have been several studies on the neurobehavioral effects of chlordecone in laboratory animals. Acute or repeated exposure to chlordecone produces observable tremor in mice (40) and rats (41) and has been quantified using a spectral analysis technique by Gerhart et al (42). Hyperexcitability or irritability has been quantified in rodents as an increased startle response (30, 43, 44). Data concerning the effects of chlordecone on learning and memory are generally lacking, but Tilson et al (44) have reported that repeated exposure to chlordecone impairs the retention of a one-way shock avoidance task in rats. Some production workers exposed to high concentrations of chlordecone were observed to be ataxic. Neuromotor deficits have also been noted in animals; mice exposed to chlordecone display motor incoordination as measured by inability to balance on a bar (45). Reiter et al (43) reported that exposure of rats to chlordecone via the diet significantly decreased activity in the open field but increased activity in a residential maze. Pryor et al (30), however, did not observe chlordecone-induced alterations in an open-field–like apparatus.

METHYLMERCURY Exposure to methylmercury has been reported to result in severe, irreversible damage to the CNS. The initial symptoms of methylmercury neurotoxicity consist of paresthesia, astereognosis, hearing and visual

disorders, cerebellar ataxia, myoclonus, irritability, and memory impairment (46–48). Some of these effects were reported to be reversible depending upon the degree of exposure. Sensory deficits reportedly occur first, with constriction of the visual field being the most common visual deficit. The motor disturbances range from impairment of fine motor control to cerebellar ataxia and dysarthria.

In rodents, the neurobehavioral effects of methylmercury consist primarily of motor effects. Several investigators have observed a progressive weakness of the hindlimbs, followed by decreases in forelimb function (49–53). Grip strength was reported to be decreased in rats chronically dosed with methylmercury (30). Horizontally directed motor activity does not appear to be markedly affected by repeated exposure to methylmercury (54, 55), although MacDonald & Harbison (56) reported decreased motor activity in mice exposed to methylmercury via drinking water. Responsiveness to noxious stimuli is reportedly intact in methylmercury-exposed animals, even in the presence of gross neuromotor deficits (50, 55). Although extensive descriptions of the neurologic effects of methylmercury have been published, changes in reactivity have not been reported (50, 57). Pryor et al (30) did not observe a significant alteration in startle responsiveness to an acoustic stimulus in methylmercury-exposed rats. Few studies have been reported on the effect of methylmercury on conditioned behaviors. Hughes et al (58) reported that prepubescent rats exposed to a single dose of methylmercury were impaired in their ability to learn an active avoidance response at 70 days of age. Beliles et al (59), using a buzzer as a conditioned stimulus, chronically exposed rats to elemental mercury vapor and found decreased pole-climb response.

ORGANOLEADS Alkylleads such as triethyl lead have been reported to produce degenerative changes in the cerebral cortex, cerebellum, and hippocampus of experimental animals (60, 61). The neurotoxic sequelae following exposure to organoleads have been well documented and include psychomotor disturbances, alterations in attention, and deficits in memory (62).

Several investigators have described the general effects of acute and short-term repeated exposure to organoleads in laboratory animals (62). In a recent paper, the effects of short-term repeated exposure to triethyl lead were quantified using several neurobehavioral tests (30). Triethyl lead was found to produce a phase of hyperexcitability characterized by increased startle responsiveness and altered responsiveness to noxious thermal stimuli, followed by a phase of hypoexcitability (63). Subsequent experiments found that triethyl lead facilitated the acquisition of a two-way shuttle box response but did not alter sensitivity to electric footshock applied to the grids of the chamber. Pryor et al (30) chronically dosed rats with triethyl lead and observed alterations in

thermal sensitivity and a trend toward impaired performance of a conditioned pole-climb response.

As a further illustration of the range of possible neurotoxic effects, Table 1 summarizes a literature survey conducted by Damstra (64) in which the reported signs and symptoms following exposure of humans to a variety of neurotoxicants are listed. It is apparent from this table that the actions of toxic agents on both the central and peripheral nervous system are numerous and complex. In addition, it is evident that chemicals having widely different structures and industrial uses can produce similar effects. Finally, it is also apparent that many of the neurotoxic effects in humans are subjective, minor, and/or nonspecific.

This brief survey of human neurotoxicology and experimental neurobehavioral toxicology is reinforcing in the sense that, for some chemicals, there seems to be a reasonable correspondence between what can be quantified in humans and what can be detected in laboratory animals. However, there are major points of disparity, particularly in regard to the description of sensory and associative or cognitive deficits in an animal model, particularly rodents. Thus, although it seems possible to develop appropriate animal models for some of the neurotoxic effects observed in humans, it is not clear that a precise one-to-one relationship is possible.

SELECTION OF TESTS FOR NEUROBEHAVIORAL TESTING

Behavior Defined

Behavior can be conceptualized as the end-product of a variety of sensory, motor, and integrative processes occurring in the nervous system (65). Operationally, behavior can be defined as the movement of an organism or its parts within a temporal and spatial context. Thus, behavior can be thought of as being comprised of units termed responses, which can be further defined as whatever covaries with effective controlling variables. By definition, an aspect of the environment that controls behavior in a functional or lawful manner is a stimulus. Behaviorists study the relationship between stimuli, behavior, and the consequences of this behavior in the environment. The behavior of an organism at any point in time is determined by the currently active environment, as well as its previous experience with these or similar environmental conditions. A change in the environment, such as the superimposition of a chemical, has the capability to change the functional relationships that control behavior.

Classification of Behavioral Tests

One way to classify behavioral procedures is to define them according to the desired level of analysis. Table 2 indicates that techniques designed to detect

Table 1 Neurotoxic effects of representative agents in humans

Representative agent	Neurotoxic effects
1. Solvents	
Hexane, acrylamide	Ataxia, tremor, paresthesia, hypersomnia, slurring of speech, delirium/hallucinations
Carbon disulfide	Anosmia, paresthesia, depression Anxiety, psychoses
2. Organochlorine insecticides (chlordecone, DDT)	Ataxia, tremor, slurring of speech, euphoria/excitement, nervousness/irritability, depression/anxiety, mental confusion, memory disorders
3. Organophosphate esters	Ataxia, paresthesia, insomnia, slurring of speech, tinnitus, amblyopia, nystagmus, abnormal pupil reactions, nervousness/irritability, depression/anxiety, psychoses, memory disorders
4. Heavy metals	
Inorganic leads	Ataxia, tremor, pathological reflexes, paresthesia, hearing loss, abnormal pupil reactions, depression/anxiety
Mercury	Facial tic, tremor, insomnia, amblyopia, depression/anxiety
Manganese	Parkinsonism, tremor, paresthesia hypersomnia, euphoria/excitement, delirium/hallucinations, memory disorders
Cadmium	Anosmia
Arsenic	Hyperesthesia
5. Organometals	
Methylmercury	Ataxia, myoclonus, paresthesia, insomnia, slurring of speech, hearing loss, abnormal pupil reactions, mental confusion

the presence or absence of toxicity are generally different from those used to determine the degree of toxicity or the lowest level of a chemical required to produce a toxic effect. Obviously, those procedures used to detect the presence or absence of an effect are usually the most frequently used to screen for potential effects. Screening procedures used routinely to assess large numbers of environmental agents are typically inexpensive to perform, do not require extensive training of either the experimental animal or laboratory personnel, and permit the assessment of large numbers of subjects. On the other hand, these procedures are usually labor intensive, frequently require subjective measurements, often yield data on less than an interval scale, and may not be as sensitive to subtle neurobehavioral deficits as more complex (i.e. secondary) techniques.

Secondary level tests are generally thought to be more sensitive to subtle changes in neurobehavioral functioning than those used for screening and may be useful in experiments concerning mechanism of action or in estimating environmentally acceptable limits. They are, however, not cost effective under most conditions.

Criteria for Test Selection

Several general considerations should be taken into account in the selection of a test for screening. Because of the large number of chemicals to be tested and the fact that relatively large numbers of animals may have to be evaluated, tests should be relatively simple to perform and require cost-effective or inexpensive equipment. Screening tests should also require a relatively short time to perform and repeat testing on an individual animal in order to assess chronic, cumulative deficits at various intervals.

Validity is another important criterion for selecting tests. Tests must be discriminant where separate functions are supposed to be under evaluation and show convergence where the same functions are being assessed. In addition, results from a test using one species should be able to be extrapolated to another species. For example, acrylamide is known to produce peripheral neuropathy in the lower limbs of exposed humans. A valid screening test should show evidence for peripheral dysfunction in the hindlimbs of animals exposed to acrylamide. Valid tests should also show as few false positives and false negatives as possible.

Another criterion is that tests should be reliable. The level of measurement associated with a particular procedure frequently determines the reliability of the technique. Nominal or categorical ratings are perhaps the most difficult to provide reliability, while procedures that yield continuous measurements are the most objective and allow for the most precise quantification of error and consequently reliability. Of course, shortcomings with some measurements can be overcome through precise standardization of test conditions and the use

Table 2 Classification of behavioral techniques

Type of test	Advantages	Disadvantages
Screening	Cost-effective, does not require extensive training of animals or personnel; permits testing of large number of animals	Requires intensive labor input; tends to be subjective and often yields less than interval scale data; may be relatively insensitive to subtle effects
Secondary	Typically automated and objective; usually provides graded data; amenable to repeated measures designs; relatively sensitive to subtle neurotoxic effects	May be costly to perform because of equipment and training of animals and personnel; may not be amenable to testing large numbers of animals

of trained raters and interrater reliability scales. Insofar as economic constraints can be satisfied, techniques producing continuous data are clearly preferred. Ordinal or ranking procedures are somewhere in between category ratings and continuous scales of measurement.

Finally, it is important that behavioral tests be reproducible. Test results should be repeatable across subjects, across samples of subjects, and across laboratories. Also contained in the concept of reproducibility is the notion of sensitivity, which concerns the ability of a test to detect effects over a range of doses and especially to detect effects at lower doses.

Strategies for Neurobehavioral Testing

Two basic approaches have been suggested for screening for neurobehavioral toxicity. Butcher (66) was among the first to describe the possible use of an apical test, a single test that requires the successful integration of intact subsystems. An example of an apical test is schedule-controlled behavior, which typically utilizes intermittent reinforcement and establishes a dependency between the occurrence of a specific response and the presentation of a specific stimulus (e.g. food). Armstrong et al (67) used a fixed interval-fixed ratio schedule to examine the effects of chronic exposure to mercury vapor in pigeons. Daily exposure to mercury resulted in an altered response rate, which occurred several weeks prior to the appearance of overt toxicity. It is interesting to note that when the pigeons were sacrificed for histopathological study, there were no microscopically detectable lesions.

Thus, an apical test such as an operant schedule of reinforcement has obvious utility in detecting the presence of toxicity and it may do so prior to the onset of structural damage. However, a major problem associated with apical tests is that performance of these tasks depends on multiple neurobehavioral processes being intact. Thus, deficits in performance may be due to alterations in any one or more neurobehavioral functions (i.e. sensory, motor, motivational, associative). Furthermore, whatever appeal the apical test has initially is immediately offset by the low probability that a single test would be generally accepted by a majority of behavioral toxicologists.

The most frequently posited strategy for assessing agents for neurobehavioral toxicity is some form of sequential assessment. The depth to which a compound is studied depends on its eventual use and the known effects. The sequential approach provides for an initial level of evaluation, the extent of which varies according to the laboratory. For example, Weiss (68) and Evans & Weiss (12) proposed a three-tier approach that consists first of observational indices of neurotoxicity. These assessments are ratings of locomotor impairment, the presence or absence of tremor, ptosis and convulsions, alterations in various reflexes, and disorders of regulatory processes. In the schema proposed by Weiss & Evans, the second phase focuses on the specific functions that

appear to be affected in the initial phase. Secondary tests assess specific sensory and motor functions using relevant state-of-the-art behavioral technology to quantify the neurobehavioral toxicity. Weiss (68) has also proposed a third phase of assessment, which focuses on human health studies.

Other investigators, such as Gad (69), have recommended an approach similar to that of Weiss & Evans, at least at the initial level of assessment. Gad proposed that a series of tests utilizing simple semi-quantitative measurements be used. These tests are in effect rating scales concerning the presence or absence of and in some cases the relative degree to which some reflexes are present. The second phase proposed by Gad consists of isolated tissue-preparation assays to establish the ability to differentiate between reversible and irreversible sensory-neural alterations.

Pavlenko (70) has also proposed three phases of testing. First, very simple methods to assess orienting and defensive reflexes, and corneal, pain, and other unconditioned reflexes are used in a tentative evaluation of potential neurotoxicity. The second phase determines the threshold and subthreshold quantities of potentially noxious substances. To accomplish this, procedures that assess higher order nervous system activity, such as conditioned reflex methods, are used. Finally, functional stress tests are employed to determine minor or latent functional changes and to study the mechanism of action of the neurotoxicant.

Reiter et al (71) have developed a Behavioral Toxicity Index based on the acute LD_{50} and experimentally derived ED_{50} values for a variety of behavioral tests. In this schema, a large index value (ratio) reflects a relatively specific behavioral toxicity and suggests that a behavioral change occurs at an exposure level below that producing overt toxicity. For the four insecticides tested (i.e. Baygon, Carbaryl, Chlordimeform, and Decamethrin), none of the four behavioral measures (i.e. figure-eight maze activity, schedule-controlled operant behavior, conditioned taste aversion, and activity in a radial arm maze) was uniquely sensitive. Ranking of the various tests overall indicated that the figure-eight maze and the schedule-controlled behavior were approximately equal in sensitivity and were more sensitive than the radial arm maze and flavor aversion procedures. The approach of using selective behavioral measures to establish a behavioral toxicity index appears to be successful, at least as it pertains to the compounds that were tested. More data are needed to determine its utility as a general screen for a wide variety of agents.

Mitchell and his colleagues (3, 14, 15) have proposed a two-tier approach to the assessment of potential neurotoxicants. Instead of relying on observational techniques at the first level of testing and then studying effects in greater detail following the detection of a behavioral change, these investigators propose judicious selection of tests at the initial phase based on several criteria. First, they propose that tests be considered for their inherent sensitivity, reliability, reproducibility, and validity. Second, tests should provide for an overview of

nervous system functioning, i.e. tests should be chosen to evaluate a wide range of functions, from "simple reflexes" to more complex functions (including sensory function, motor strength, and coordination), reactivity (excitability), and associative factors. Recently, Pryor et al (30) reported on the results of a three-year study comparing the effects of eight chronically administered neurotoxicants (acrylamide, arsenic, chlordecone, methylmercury, monosodium salicyalte, lead acetate, triethyl lead, and tetraethyl tin) using a battery of tests (i.e. motor activity, startle to an air puff and acoustic stimulus, fore- and hindlimb grip strength, negative geotaxis, performance on a multisensory conditioned poleclimb shock-avoidance task, and body weights). For the compounds tested, these investigators found a reasonably good association between signs of neurotoxicity reported in humans and those neurobehavioral effects predicted in rodents.

EXAMPLES OF METHODS TO BE USED IN TESTING

Tests of Motor Function

Spontaneous motor activity in rodents has been extensively used in behavioral toxicology (2, 72). Movement within the living space or environment is a high probability response in animals and can easily be manipulated by environmental changes, including exposure to neurotoxicants. Although seemingly simple, locomotor activity is a very complex behavior comprised of a variety of motor acts, such as horizontally and vertically directed movement, sniffing, and grooming. Rating scales have been developed to fractionate motor activity into its relative components (73). The measures used most often in behavioral toxicology are horizontally and vertically directed activity (72). A large variety of devices, automated and unautomated, have been invented to measure motor activity, and quantitatively and qualitatively different effects can be observed following exposure to a neurotoxicant depending upon the apparatus used. Although it is premature to conclude that one technique or procedure is better than another, the figure-eight maze as utilized by Reiter and colleagues (2, 72) has been used extensively and successfully to detect effects produced by a number of chemicals.

Although Reiter and colleagues (2) have used the figure-eight maze as a residential maze to measure chemical effects on diurnal activity patterns, their most recent research has almost exclusively employed assessment during shorter time intervals. Elsner et al (74) have also reported a method for continuous monitoring of spontaneous locomotor patterns of rats. Using computer-assisted techniques, these investigators found that methylmercury treatment lowered activity during the night portion of the diurnal cycle.

Before going on to discuss other measures of motor function, some comment should be made concerning the meaning of data generated by procedures utilizing motor activity measurements. In some respects, motor activity can be

viewed as an apical test (see above). As pointed out by Reiter (2), activity is not a unitary measure and a change in the frequency of this behavior could reflect toxicant-induced changes in any one or more sensory or motor functions, alterations in reactivity (excitability) or motivational states, or perturbations of a variety of regulatory states (i.e. diurnal cycles, energy balance of the animal). Thus, if a change in motor activity is observed, then additional tests are needed to determine the cause (i.e. is there a decrease in activity because the animal is paralyzed or is it because there is so much liver damage that the animal suffers from "general malaise").

Many types of screening tests are currently used to assess the effects of neurotoxicants on motor function. The most simple of these include observational assessments of body posture, muscle tone, equilibrium and gait, and righting reflexes (53, 69, 75). These tests are quantal or categorical at best and are generally subjective in nature.

A variety of other techniques have been developed to evaluate motor function in a less subjective fashion, including performance on a rotating rod (76), inclined screen, or plane (77); swimming to exhaustion (78); or suspension from a horizontal rod (79). One technique used with increasing frequency is quantification of hindlimb splay; Edwards & Parker (35) placed ink on the feet of rats, dropped them from a specified height, and measured the distance between the marked digits. Schallert et al (80) used a similar technique of inking the paws to evaluate abnormal gait in rats treated centrally with 6-hydroxydopamine.

Neurotoxicant-induced alteration in motor coordination has been evaluated by Pryor et al (30) using a negative geotaxis procedure, while the hindlimb extensor reflex was quantified using a method described by Cabe & Tilson (81). Grip strength is a frequently reported neurological sign in humans and fore- and hindlimb grip strength in rats and mice has been quantified using commercially available strain gauges (82).

One common neurological effect observed in animals exposed to neurotoxicants is tremor. A number of rating scales and semiquantitative procedures have been reported to measure tremor (42). Recently, a simple but expensive spectral analysis technique was reported that permits the rapid evaluation of tremor in freely moving animals (42).

More complicated techniques have been devised to measure motor deficits in laboratory animals. For example, Falk (83) used operant conditioning procedures to train animals to press a lever within a designated range of force for a given period. Falk and others (84) have used this procedure to study the effects of agents on fine motor control.

Tests for Sensory Function

Alterations in sensory processes, such as paresthesias or visual or auditory impairments, are frequently among the first signs of toxicity produced in

humans exposed to toxicants. The most sensitive methods used to detect sensory deficits in animals involve psychophysical measurements to arrive at some estimation of the ability of an organism to make a differential response in the presence of a stimulus varied across some physical dimension (85). However, the great majority of psychophysical studies have been done in nonhuman primates and avian species rather than rodents.

One of the least complex approaches to the study of sensory deficits is based on the localization or orientation response. Marsnall and his colleagues (86–88) have described a battery of observational tests in which a stimulus (a square of paper brought in from behind the animal's head over either eye, clicks presented close to and behind the ear, presentation of a strong odor on each side of the head, or pressing hairs calibrated to bend at a given force along various portions of the body surface) is presented and the presence or absence of a localization or orientation response to the source of the stimulus is recorded. Such techniques have been used to demonstrate sensory inattention as well as hyperexcitability in rats having lesions in various regions of the brain. Pavlenko (70) has described a variety of stimulus-elicited orientation reflexes used in the Soviet Union.

In spite of the fact that observational tests are simple to perform, they are labor intensive, especially if interrater reliability scales are used. Moreover, the scoring of the tests is frequently subjective and necessitates testing under blind conditions. Finally, the data are usually of a quantal nature (i.e. the response is scored as being either present or absent) or categorical (rating scores); thus, interpretation of the results is difficult, particularly in repeated measures designs.

Several attempts have been made to develop simple yet objective tests for sensory dysfunction in rodents. For example, depth perception has been assessed using a visual cliff procedure, which determines whether or not an animal will choose to step onto a nearby platform or floor ("shallow" floor) compared to one that may be perceived as being further away ("deep" floor) (89). Another simple test of visual function is the optokinetic drum, which relies on the optokinetic nystagmus or optomotor response (i.e. tracking a moving object with the eyes and head for a certain distance until the head is repositioned back into the frontal plane). This measure is believed to assess visual acuity (90), although there are little data reported using this technique to study toxicants. One screening test used by some investigators to assess hearing is the acoustic startle reflex, which consists of measuring the presence (and magnitude) or absence of the response to the presentation of a novel sound or tone (91). Pain sensitivity can be assessed using standard psychopharmacological techniques such as the tail flick or hot plate (30), while taste reactivity has been assessed using taste-aversion procedures (92).

Several more complicated paradigms have been proposed to assess sensory

dysfunction in rodents. For example, maze and similar types of apparatus are frequently used to test for alterations in the performance of tasks based upon discrimination of sensory cues (93). On the basis of work done using visual cues, as well as work done with other sense modalities such as somesthesis (94, 95), mazes and maze-like apparatus appear to have some utility in evaluating sensory deficits in rats. However, as pointed out by Evans (93), care must be taken to determine the relative contribution of motor and higher level functions when interpreting results from such experiments.

One of the more objective ways to evaluate sensory deficits involves the use of operant technology. In these experiments, an animal is motivated by food or electric shock to make a response, such as a lever press. Eventually the animal learns to make the response only under certain stimulus conditions and, by varying the parameters of the stimuli, a graded stimulus-intensity response function curve can be determined. Of course, neurotoxicants with specific sensory effects would be expected to alter this curve. Auditory loss has been reported following exposure to kanamycin using such techniques (96, 97). Besides the procedures mentioned for auditory deficits, operant techniques have been used to study the effects of chemicals on light flicker discrimination (98), olfactory cues (99), and reactivity to electric shock (100).

Recently, Pryor et al (30) reported on the use of a multisensory conditioned avoidance response (CAR) to assess three sensory modalities concurrently in the same animal. In this procedure, a rat learns to climb or pull a rope to escape and then to avoid a noxious (1 mA) electric footshock. Eventually, the response is brought under the control of three conditioned stimuli (a 4-kHz tone, a low-intensity nonaversive current on the floor, .125 mA, and a change in the intensity of the chamber house light). Three intensities of each stimulus are presented during each series of trials on each day of testing, thereby permitting a quasipsychophysical response function to be generated. Once the animals are trained to make a response, they are exposed to a variety of toxicants and the change in response measured. A specific sensory neurotoxicant should have a specific effect on avoidance behavior controlled by one of the respective stimuli. Experiments with positive control manipulations (i.e. constant light in the home cage to alter visual function or repeated administration of kanamycin to produce hearing loss) have successfully demonstrated the validity of the technique. In a recent paper, Pryor et al (101) reported functional hearing loss associated with hair cell damage in the inner ear in rats exposed repeatedly to toluene.

Tests for Reactivity or CNS Arousal

One of the frequently reported indicators of neurotoxicity is nervousness and irritability (or, on the other hand, hypersomnolence and lethargy). One procedure frequently used to test for general responsiveness to environmental stim-

ulation is the startle reflex. According to Davis and colleagues (91, 102), the startle reflex is mediated by a five-synapse pathway beginning at the auditory nerve and ending in the lower motor neurons. By using a supramaximal novel stimulus, a response having a short, reproducible latency occurring in virtually every animal can be elicited. The startle response can be influenced by changing the parameters of the eliciting stimulus and therefore has utility in psychophysical studies (see above).

Changes in the magnitude of the startle response have been associated with toxicant-induced alterations in CNS excitability. Increases [for chlordecone, see (43, 103)] and decreases [for PBBs, see (104)] have been reported. The startle response appears to be a relatively good indicator of general responsiveness when used under the appropriate conditions. However, other measures of neurological function should be considered when studying the startle response, since it is a reflex that contains a neuromuscular component.

Another procedure commonly used to determine changes in the excitability of the nervous system is to study alterations in responsiveness to chemically or electrically induced seizures. For example, changes in the threshold to produce maximal electroshock seizure (MES) have been shown in rats exposed during development to lead (105). Dyer et al (106) have shown that rats exposed to trimethyl tin were more sensitive to the effects of pentylenetetrazol, suggesting a general increase in seizure susceptibility.

Learning and Memory

The ability to learn and remember has obvious adaptive value for an organism. The capacity to learn permits an organism to escape or avoid aversive situations or approach desirable objects and to store the memory of these contingencies for use at some future time. The specificity of a toxic effect on learning must be interpreted within the context of the experimental design and in conjunction with controls that assess toxicant-induced effects on sensory, motor, and motivational processes.

Behavioral toxicologists have used a variety of experimental paradigms to assess learning/memory in laboratory animals. Briefly, procedures have been designed to determine acquisition using positive as well as negative reinforcing contingencies and to assess intermediate and long-term memory. A few working procedures have been developed to measure the ability to adjust to a new contingency once an initial task has been learned.

PROCEDURES USING NEGATIVE REINFORCEMENT Aversive conditioning utilizing electric footshock has been used frequently in behavioral pharmacology and toxicology. Passive avoidance procedures involve training an animal to withhold a response to avoid being shocked. For example, Mactutus et al (107) used a multiple measure step-through passive avoidance procedure to assess

learning/retention deficits in rats exposed neonatally to chlordecone. What is unique about the protocol they employed is that several measures (i.e. initial step-through latencies, frequency of head pokes, half crosses and full crosses, as well as the traditional latency to reenter the chamber after being shocked) were taken. In addition, retention over several time points (immediately and 72 or 144 hours after training) was assessed. A similar procedure has been used to assess retention deficits after trimethyl tin administration to adult rats (108) and following intraventricular administration of the cholinergic cytotoxicant AF64-A (109). The passive avoidance procedure has the advantage of being rapid and easy to perform without requiring extensive resources; it has the disadvantage of producing highly variable results if performed under inadequate test conditions or if the appropriate retention intervals are not used. Finally, we highly recommend that measurement be made on the nonassociative variables mentioned above, as well as evaluation of alterations in motivational factors, such as changes in pain thresholds to footshock.

One-way shock avoidance tasks require the animal to move from one compartment to another in order to escape or avoid being shocked. Once the response is made, the animal is placed back in the original compartment and the process is repeated. In one-way avoidance, the animal does not have to learn to return to the physical space where it has just been shocked. Using this type of test, Tilson et al (44) reported that rats exposed chronically to chlordecone learned to avoid as rapidly as controls, but displayed a significant retention deficit when retested several days later.

Another variant of the shock-motivated learning tasks is the two-way shuttle box paradigm. In this procedure, rats learn to shuttle from one compartment to another in order to escape or avoid electric footshock; however, unlike one-way avoidance, the animals have to learn to return to a compartment where they have just been shocked. It is interesting to note that differences in effects can be made between the one- and two-way procedures. For example, Sobotka et al (110) reported that rats exposed neonatally to lead performed as well as controls on a one-way shock avoidance task, but displayed significant deficits when required to learn a two-way avoidance task. Moreover, rats exposed to chlordecone chronically did not show deficits in two-way avoidance (R. Squibb, T. Burne, H. Tilson, unpublished observations), in spite of previous reports that similar exposure to chlordecone affected one-way avoidance (44).

In general, one- and two-way avoidance tasks are conducted in one training session or over several discrete massed trials, while retention is assessed in one or more trials at some point following demonstration of learning. A somewhat more complex learning task than either the one- or two-way avoidance procedures is the symmetrical Y-maze. In this procedure, a light or tone is activated in one of two arms not occupied by the animal, which has a predetermined amount of time to run to the proper arm of the maze or it will receive an electric

shock. Not only does the animal have to learn when to run, but it must also learn where to run (111). The Y-maze has been used successfully by Vorhees (112) to study learning ability of rats exposed in utero to vitamin A.

The preceding techniques have all employed electric footshock as a negative reinforcer to promote learning. One procedure that uses another approach is the water maze. Animals are placed into a maze full of water and are required to learn a series of correct turns in order to gain access to an exit ramp. Learning trials are preceded by straight channel swimming trials as an adaptive procedure and to determine if there are measurable neuromotor deficits. Vorhees et al (113) detected learning deficits in animals exposed during development to vitamin A using a Biel water maze, while Zenick and his colleagues (114, 115) have found learning deficits in animals exposed to lead or mercury during development.

PROCEDURES USING POSITIVE REINFORCEMENT Another approach to assessing learning abilities in animals is to use positive reinforcement, such as a food reward for a previously food-deprived animal. Mazes and similar apparatus have been used to demonstrate that animals will learn to make a response (i.e. move to a specific location or press a lever) to receive reinforcement. One procedure using positive reinforcement that shows promise in behavioral toxicology is the radial-arm maze (RAM). The RAM is a complex spatial learning task in which animals must "remember" a list of previously entered and non-entered feeders during a free-choice test session (116, 117). The most commonly used RAM consists of a circular arena from which eight equidistant arms radiate like spokes from a wheel. Each of the eight arms is baited at the beginning of a test session; the trial ends following consumption of all available pellets or after a fixed period of time has elapsed. The most effective strategy for solving the maze is to enter and eat in each of the arms only once. Recently, Walsh et al (108) reported that trimethyl-tin–exposed rats display impaired performance in this task and that the behavioral deficit may be due to an alteration in the integrity of limbic forebrain structures such as the hippocampus.

Another positive reinforcement procedure developed recently is the two-choice visual discrimination task described by Tilson et al (118). In this task, animals are trained in a discrete trial task to make a nose-poke-operant response in the presence of a visual cue located on one side of a cue panel. After the animal learns the correct discrimination, which occurs over a period of days, the contingency is reversed, i.e. a response to the side in which the cue lamp remains unlit is reinforced. Tilson et al (118) found that rats exposed to chlordecone during development showed a trend toward improved acquisition and were markedly different from the control rats in the way that they responded during the reversal phase of the test.

Naturally Occurring Behaviors

A general principle in psychology and animal behavior is that the behavior of an organism is likely to be stable and predictable given stable environmental conditions. Behaviors such as those that occur naturally in the home cage, such as eating, drinking, and general locomotor activity, are related to the ecological and evolutionary history of the organism.

One approach to the use of naturally occurring behaviors is to measure the repertoire of animals in their laboratory environment, i.e. in the homecage. Locomotor activity, food intake, and water intake can be easily quantified (119) and, once a stable pattern of homecage activity is established, toxicants can be introduced and the resulting alterations in behavior measured (3).

One naturally occurring response that has been proposed to screen for toxicants is geophagia, which is the ingestion of nonnutritive substances following conditions known to produce illness (120). Closely associated with geophagia is the conditioned taste aversion procedure. When rodents are given access to a flavored solution followed by exposure to a drug or toxicant, they often display an aversion upon subsequent presentation of the solution. Flavor aversions have been reported to occur following a number of environmentally relevant chemicals, such as arsenic, cadmium, copper sulfate, lead, methylmercury, chlordimeform, 2,4,5-T, acrylamide, physostigmine, and alkyltins (121, 122).

Silverman and colleagues (18, 123) have been proponents for the study of social behaviors in animals exposed to chemicals. These investigators record species-specific behaviors (i.e. exploration, sex-related activities, aggression, submission, etc.) of rats in an observation cage and measure toxicant-induced changes in the frequency of the behaviors. Through laborious and time-consuming observations and recordings, Silverman et al (123) were able to see significant changes in behaviors of animals exposed to methyl mercury via the diet (social behavior was decreased after 16–17 days of exposure to 75 ppm).

Alternative Approaches

Behavioral procedures described thus far have focused primarily on methods employed by researchers in Western countries. There is an equally important and vast literature on approaches used by investigators influenced by Sechenov and Pavlov (124, 125). In general, the Soviet Union and other countries pose the problems of neurobehavioral assessment in terms of physiological aspects of nervous system function.

As discussed earlier, Pavlenko (70) proposed simple tests of unconditioned responses as the first tier in neurobehavioral screening; tests of conditioned reflexes were proposed for later study. These tests involve various conditioned reflexes, such as those requiring shock or food motivation and induction processes of learning and acquisition. Although much of the terminology of the

Pavlovian-oriented researchers differs from that of the more behaviorist framework of the West, there is considerable convergence in the two approaches (12). Where they may differ is in how a behaviorally toxic effect is interpreted and later translated into regulations for the environment.

PROBLEMS AND RESEARCH NEEDS

Summary of Purpose and Scope

Numerous methods are available for use in toxicity assessment, yet several problem areas need to be resolved before there can be any agreement on a screening program. One significant question concerns the perceived goal of a screening program. It seems imperative that the large number of new chemicals introduced each year and the thousands of agents already in the environment need to be assessed for their effects on the nervous system. Neurobehavioral effects are significant in that, in some cases, a functional deficit may be observed prior to the onset of a more obvious toxic effect or before there is morphological damage.

If it is accepted that behavioral techniques have a place in the screening of toxic agents (along with those of other disciplines, especially neurophysiology), the next question concerns the choice of tests to be included in the screen. The purpose of the neurobehavioral screen is to provide an initial evaluation of the effect of agents on behavior and the nervous system so that neurotoxic agents will not reach the marketplace without proper warning. Thus, it is important that the tests used in the screen be sensitive, reliable, and reproducible as well as cost effective. It is important to realize that if insensitive techniques are used or if toxic effects observed in one laboratory cannot be reproduced in another, then resources have been wasted. More importantly, an insensitive screen could allow potentially neurotoxic agents to enter the environment. It is logical to assume that tests that are objective to perform (automated as much as possible) and yield continuous level data for analysis will contribute to the sensitivity and reliability of a battery of tests. Standardization of the protocols and conditions under which tests are performed will greatly assist in meeting the objectives of reliability and reproducibility.

Another objective in establishing criteria for neurobehavioral screening is that they should have some relevance to human toxicosis. One obvious way to accomplish this is to include a variety of tests that attempt to assess the wide range of signs typically observed in humans exposed to neurotoxicants. One approach toward this objective is to choose animal tests whose results can be applied to human neurobehavioral functions. Validation of test methods in neurobehavioral screening should be an empirical matter. Mitchell and his colleagues (3, 15, 16) have argued that validation of sensitive and reliable methodologies in animals (rats) is possible by first comparing compounds known to have specific neurotoxic effects in several tests chosen to overlap in

terms of signs evaluated. The profile generated by such a comparison could be used to evaluate the sensitivity and selection of those tests assumed to measure the same neurobehavioral functions. This strategy permits test validation by showing the similarities between procedures assumed or purported to evaluate the same function and by providing a distinction between procedures assumed to measure different processes.

Problems Inherent in Behavioral Tests

One problem with the use and therefore selection of behavioral tests for screening is that it is often difficult to isolate the relative contributions of the various sensory, motor, arousal, or associative factors that may contribute to a behavioral toxic effect. For example, if a toxicant produces a decrease in the probability of a simple reflex, such as the orientation response to an auditory stimulus, the conclusion that hearing is affected depends upon concurrent measurement of motor dysfunction or responsiveness of the animals to nonaural environmental stimuli. In effect, a profile of neurobehavioral effects may be required in order to demonstrate the specificity of the neurotoxic lesion.

Another problem is that, with repeated exposure to a chemical, animals can adapt to its effects. As Norton (61, 125) points out, the effects of repeated exposure to a chemical may go undetected because of homeostatic mechanisms triggered by the presence of the agent. Furthermore, many organs possess an excess capacity that can be damaged and go undetected by functional tests. The so-called functional reserve of the central nervous system should be considered in the selection of tests for screening, perhaps by incorporating in the test procedures one or more conditions in which the system(s) or organism(s) is placed under some sort of stress. The combination of the test substance plus stress may result in a greater or detectable deficit in neurobehavioral function. Examples of stressors that have been used are ethanol and other drugs, muscular or work stress, exposure to cold, and auditory and electrical stimuli (70, 126).

Another problem that should be considered is that seemingly similar methods might yield different results. For example, Reiter et al (127) used two measures of locomotor activity (open field and residential maze) to evaluate the toxicity of chlordecone and triethyl tin. Chlordecone produced a dose-related decrease in the open field, whereas a dose-related increase was observed in residential maze activity. With triethyl tin, there was a dose-related decrease in residential maze activity but no change in activity in the open field. Thus, there were qualitative and quantitative differences in the two measures of locomotor activity.

Research Needs

In addition to the need for standardization and validation of tests discussed above, more emphasis needs to be placed on determining subpopulations at

greatest risk. Most of the data in behavioral toxicology have been generated with young adult male animals. More emphasis needs to be placed on comparative effects in animals that may be predisposed to toxic insult (i.e. the very young, the elderly, the malnourished, females versus males).

Another area that deserves attention concerns the potential interactions of two or more chemical agents. In the environment, the probability of exposure to several toxicants exceeds the probability of being exposed to a single agent (i.e. Love Canal in the US). Although it is preferable to know more about the potential effects of toxicants when they are administered singly before studying the problems of mixtures, the reality of the environmental situation suggests that a strategy for the evaluation of mixtures be considered.

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