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Preface

This monograph is the result of a technical review meeting that was held May 20-21, 1991. The National Institute on Drug Abuse (NIDA) supports neurotoxicity research as part of its mandate to explore the consequences of drug abuse. This research has provided important data on the neurotoxicity of several abuse substances, such as amphetamine and its congeners; tetrahydrocannabinol, the psychoactive component of marijuana; and phencyclidine. These NIDA-supported studies also have served to further public health on a broader scale by leading to the development of new methodologies for studying neurotoxicity and by elucidating general principles that will guide future research in neurotoxicology.

During the course of the technical review meeting, a consensus was reached that no one technique could be used to define a neurotoxic effect but, rather, that confluent data from a variety of tests are needed to establish neurotoxicity. A variety of different methodologies were presented, although by no means all those being employed in the field. In general, the methodologies could be divided into those for screening or hazard identification and those for characterizing neurotoxicity, including studies of mechanism of action. Screens for evaluating large numbers of compounds are of great value to regulatory scientists and are useful in the drug abuse field to evaluate new drugs of abuse. Several presentations focused on screening methodologies based on behavior, neurophysiology, neuroanatomy, or neurochemistry. Other presentations focused on different methodologies designed to study underlying mechanisms of neurotoxicity. A knowledge of the basic mechanisms of neurotoxicity may help researchers understand some of the effects of drugs of abuse and may provide useful models for studying neurodegenerative diseases.

At the meeting, Dr. John Olney presented a paper on MK801 neurotoxicity, but time constraints prevented him from writing a chapter for this monograph. To get a sense of the content of Dr. Olney's talk, I recommend the paper by Olney, J.W.; LaBruyere, J.; Wang, G.; Wozniak, D.F.; Price, M.T.; and Sesma, M.A., "NMDA antagonist neurotoxicity: Mechanism and prevention," *Science,* 254, 1515-1518, 1991. In addition to presenting different methodologies, Dr. Olney and colleagues pointed out the importance of understanding the neural circuitry mediating neurotoxic effects.

A number of neurotoxicologists and neuroscientists who did not present papers were active participants in this meeting and contributed to the lively discussion following each talk. Unfortunately, there is no roster of meeting participants, so I cannot thank each person individually. In transcribing the discussion, where possible I have identified the questioner. Several participants were identified by name on the tape, and they are Tom Sobotka, Ph.D., and Nate Appel, Ph.D., of the US. Food and Drug Administration; Diane Miller, Ph.D., of the U.S. Environmental Protection Agency; and Rona Ramsay, Ph.D., of the University of California, San Francisco. I think the discussions were exceedingly valuable, and they have been included following each chapter. Because of taping failure, the discussions following the presentations by Dr. O'Callaghan and Dr. Cho were lost.

I hope that the readers of this monograph get some sense of the enthusiasm of the participants and of the spirited discussions that took place on the state of the science. All the authors have provided superb chapters that should stimulate readers to think about the methodological issues raised in this monograph.

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Neurobehavioral Methods Used in Neurotoxicology

Hugh A. Tilson

INTRODUCTION

Neurotoxicity has been defined as any adverse effect on the structure or function of the central and/or peripheral nervous systems produced by a biological, chemical, or physical agent (Tilson 1990a). Historically, toxicity has been defined in anatomical or structural terms (Weiss 1988). It is now generally accepted that neurotoxic effects can be identified and characterized at one of many levels of organization of the nervous system, including the chemical, anatomical, physiological, and neurobehavioral levels. Recently, there has been an increased reliance on neurobehavioral indices of neurotoxicity to detect and characterize neurotoxicity (Weiss and Laties 1975; Zbinden et al. 1983; Annau 1986; Tilson 1987, l990b; Cory-Slechta 1989). It has been suggested that because behavior is the net result of integrated sensory, motor, and cognitive function occurring in the nervous system, chemical-induced changes in behavior may be a relatively sensitive indicator of nervous system dysfunction (National Academy of Sciences 1975). In some cases, the dose-effect curve for neurobehavioral measures may lie to the left of other endpoints of toxicity. As pointed out by Weiss (l988), neurobehavioral assessments are important because it is often the case that one of the earliest indications of exposure to neurotoxicants is subtle behavioral impairment such as paresthesias or short-term memory dysfunction; frequently, such behavioral effects precede more obvious and frank neurological signs. Furthermore, neurobehavioral measures are generally noninvasive, facilitating the study of the onset and duration of neurotoxicity.

One measure of the acceptance of neurobehavioral measures in environmental toxicology is their increased use in the regulatory context. Since 1975, several expert panels or committees have recommended that neurobehavioral tests be used in the assessment of chemicals for potential neurotoxicity (Reiter 1987; Tilson 1990a). As early as 1975, Great Britain and Japan began requiring neurobehavioral testing of animals to evaluate the developmental neurotoxicity of new drugs (Kimmel 1988). In 1983 the European Economic Community adopted testing protocols similar to those required by Great Britain and Japan (Kimmel 1988), and in 1984 the World Health Organization published proposed

testing guidelines for drugs and other agents (World Health Organization 1984). The Organization for Economic Cooperation and Development is now considering several neurotoxicity testing protocols, many of which involve neurobehavioral assessments, In the United States, regulatory agencies utilize neurobehavioral data in the premarket approval of several different kinds of chemicals. For example, the Food and Drug Administration requires a number of clinical observations in the routine testing of drugs (Tilson 1990*a*). Neurobehavioral data have been used to set threshold limit values of exposure to many chemicals in the workplace (Anger 1984). In addition, the Environmental Protection Agency (EPA) has published several testing guidelines covering the use of various neurobehavioral endpoints, including a functional observational battery (FOB), motor activity, and schedule-controlled behavior (Tilson 1990a). Neurobehavioral endpoints are also included in EPA testing guidelines for organophosphate-induced delayed neuropathy and developmental neurotoxicity.

DEFINITION AND USE OF NEUROBEHAVIORAL ENDPOINTS IN TOXICOLOGY

Behavior can be defined as whatever an organism is doing (Cory-Slechta 1989). Many behavioral neurotoxicologists classify behavior as being either respondent or operant (Tilson and Harry 1982; Cory-Slechta 1989). Respondent behaviors generally occur in close temporal contiguity with an eliciting stimulus. Operant behavior is emitted and occurs within the context of many environmental stimuli but is not elicited by any one stimulus.

Behaviors also can be learned or conditioned. Most behavioral scientists recognize at least two types of processes associated with learning: classical and instrumental conditioning. Classical or respondent conditioning involves the pairing of two stimuli, one of which elicits a reflex and one of which is neutral. With repeated pairing of the two stimuli, the previously neutral stimulus becomes a conditioned stimulus and elicits the response in the absence of the original eliciting stimulus, Instrumental or operant conditioning involves the repeated presentation or removal of a stimulus following a behavior to increase the probability of the behavior (i.e., reinforcement). A reinforcer is a stimulus that increases the probability of behavior. If the probability of a behavior goes up following the presentation of some stimulus, then positive reinforcement has occurred. If the probability of a behavior goes up after the removal of a stimulus, then negative reinforcement has occurred.

The use of neurobehavioral procedures in environmental toxicology varies according to their intended use (National Academy of Sciences 1975). Several expert panels have recommended a tiered approach to the evaluation of chemicals for neurotoxicity (Reiter 1987; Tilson 1990*a*). Each stage of assessment incorporates decision points as to whether available information is sufficient for determining the neurotoxicity of a chemical. Procedures

used in later stages of assessment study more specific questions about the neurotoxicity of a chemical. Such a strategy typically begins with simple, rapid, inexpensive methods to detect the presence of neurobehavioral toxicity. Examples of such tests include simple measures of locomotor activity, sensorimotor reflexes, and neurological signs. Studies concerning biological mechanism(s) of action or providing data to determine a no-observable-adverse-effect level or lowest-observed-adverse-effect level involve more sensitive tests and special equipment. Examples of techniques used in later stages of assessment include discriminated conditioned response techniques to assess sensory or motor dysfunction and most procedures to evaluate cognitive function.

Neurobehavioral tests that are used to assess chemical-induced changes in neurobiological function in animals are frequently based on human neurotoxicity. Table 1 summarizes the various types of signs and symptoms reported by humans exposed to a wide range of neurotoxicants (World Health Organization 1986; Tilson and Mitchell 1984). Chemicals can cause a diverse range of neurobiological effects in humans, including sensory, motor, autonomic, and cognitive dysfunction (World Health Organization 1986). Clinicians also recognize sensorium changes in humans, including irritability, attention

TABLE 1. *Examples of signs and symptoms reported by humans exposed to neurotoxicants*

Function	Manifestation
Sensory	Changes in smell, vision, taste, hearing, balance, proprioception, feeling, pain
Motor	Weakness, decreased strength Tremor Incoordination or ataxia Abnormal movements, including myoclonia, fasciculations, behavioral seizures Hypermotor or hypomotor activity
Autonomic	Body temperature changes, “cholinergic crisis”
Cognitive	Learning, memory deficits
Sensorium	Hallucinations, delusions, apathy, stupor, coma

SOURCE: Adapted from World Health Organization 1986 and Tilson and Mitchell 1984

difficulties, hallucinations, dementia, depression, stupor, and coma. Table 2 summarizes several neurobehavioral endpoints that have been used to quantitate neurotoxicity in animals. Many neurobiological deficits described in humans exposed to neurotoxicants can be quantified objectively in animal models. In the following sections of this chapter, several procedures used in animal neurobehavioral toxicology to assess neurotoxicant-induced alterations in sensory, motor, and cognitive function are described. Selection of the tests for discussion is not meant to be inclusive but rather to provide examples of the utility of different kinds of neurobehavioral tests.

EXAMPLES OF ANIMAL NEUROBEHAVIORAL TESTS IN TOXICITY SCREENING

Functional Observational Batteries

Procedures used to identify the presence of neurotoxicity are usually designed to test many animals and are not considered to be as sensitive to subtle effects as

TABLE 2. *Examples of behavioral endpoints of neurotoxicity in animals*

Measurement	Effect
Sensory	Alterations in visual, auditory, gustatory, somatosensory, and pain sensations
Sensorimotor reflexes	Absence or decreased occurrence, magnitude, or latency; potentiated reflex
Neuromuscular impairment	Altered grip strength, hindlimb splay
Motor activity	Increases or decreases
Abnormal motor movements	Incoordination, ataxis, abnormal gait, dystonia, myoclonia, tremor, retropulsion, behavioral seizures
Autonomic dysfunction	Piloerection, salivation, increased urination, diarrhea, hypothermia
Cognitive deficits	Alterations in learning or memory
Schedule-controlled behavior	Change in rate or temporal patterning of responding

more specialized tests for neurobiological dysfunction. The use of an FOB for screening is now generally accepted. Several batteries for rodents (Gad 1982; Moser et al. 1988; Haggerty 1989; O'Donoghue 1989; Moser l988), dogs (Schaeppi and Fitzgerald 1989), and nonhuman primates (O'Keeffe and Lifshitz 1989) have been developed to assess major overt behavioral and neurological effects. FOB evaluations in animals are similar to clinical neurological examinations in humans in that they rate the presence and, in some cases, severity of specific neurological signs.

An FOB typically assesses several neurobiological domains, including neuromuscular (i.e., weakness, incoordination, gait, and tremor), sensory (i.e., audition, vision, and somatosensory), and autonomic (i.e., pupil response and salivation) functions (table 3). FOB data may be interval, ordinal, or continuous, and test results are judged according to the number and type of signs affected, the dose(s) at which neurotoxic signs are observed, and the persistence of such effects. FOB assessments are usually performed in a sequential fashion, proceeding from the least to the most interactive with the animal. An FOB can provide data concerning dose-response and onset, duration, and persistence of an effect; can differentiate neurotoxicants from nonneurotoxicants; and can differentiate neurotoxicants having different mechanism(s) or site(s) of action.

The application of an FOB to assess neurotoxicants generally results in a relatively large data set. In a recent paper, Moser (1991) described an approach

TABLE 3. *Behavioral endpoints used* in *a functional observational battery*

Home Cage and Open Field	Manipulative
Posture	Ease of removal
Convulsions and tremors	Ease of handling
Palpebral closure	Approach response
Lacrimation	Auditory startle
Piloerection	Response to aversive stimulus
Salivation	Righting reflex
Vocalizations	Landing foot splay
Rearing	Grip strength
Urination and defecation	Pupil reflex
Gait	
Arousal	
Mobility	
Stereotypy	
Bizarre behavior	

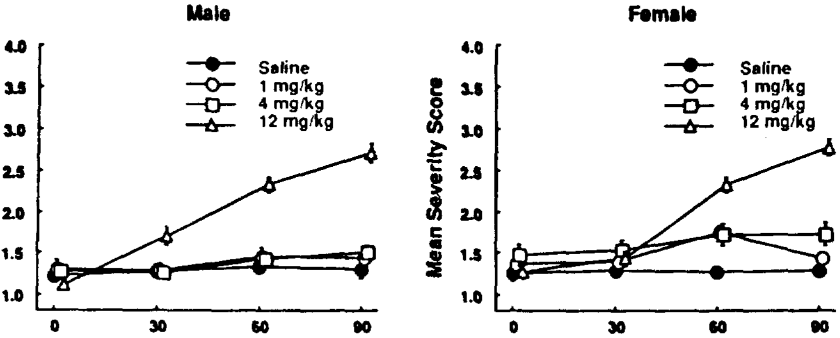
SOURCE: Adapted from Moser 1989

to summarize FOB data from neurotoxicity experiments. Rats were dosed with acrylamide or 2-hydroxyethyl acrylate (HEA) and assessed for neurotoxicity after 30, 60, or 90 days of dosing. Moser (1991) normalized all the data from each rat for the 29 measures in the FOB on a scale of 1 to 4. The normalized scores were then summed for each animal within measures assigned to one of six functional domains (i.e., autonomic, central nervous system (CNS) activity, CNS excitability, neuromuscular, sensorimotor, and physiological). Using this approach, acrylamide-treated rats showed statistically significant effects only in the neuromuscular domain, which consisted of six different tests, including gait score, mobility score, landing foot splay, forelimb and hindlimb grip strength, and righting reflex. HEA had no significant effects on any of the functional domains. Figure 1 shows the average neuromuscular scores for male and female rats exposed to acrylamide or HEA for 90 days. Routine analysis of variance was used to show a significant treatment by time interaction. Male and female rats were significantly affected after 90 days of dosing with 12 mg/kg of acrylamide. Figure 1 also shows that HEA had no effects on neuromuscular function at any time during dosing. The strategy of normalizing and summarizing FOB data into functional domains is a promising advance in the routine analysis of screening data from studies using FOB.

Motor Activity. Motor activity includes a broad class of behaviors reflecting the net integrated output of the sensory, motor, and associative processes of the nervous system (Reiter and MacPhail 1982). Motor activity is usually quantified as the frequency of movements over time. Effects of chemicals on motor activity are usually expressed as total number of counts or as a percentage of some control value (i.e., control group or preexposure baseline). The frequency of motor activity within a test session usually decreases, a phenomenon known as habituation.

Automated procedures to assess motor activity have been used to examine the effects of psychopharmacological and neurotoxic chemicals (Reiter and MacPhail 1982; MacPhail et al. 1989). Although it is generally accepted that motor activity measurements are sensitive, reliable, and efficient (MacPhail et al. 1989), it has been argued that motor activity measurements alone lack specificity and do not easily differentiate the effects of psychoactive and other chemicals from neurotoxicants (Maurissen and Mattsson 1989). However, motor activity procedures are frequently used in conjunction with other screening tests such as the FOB. One major conclusion from motor activity is that the acute effect of most chemicals is a depression of motor activity. However, some neurotoxicants (i.e., trimethyltin (TMT), triadimefon, triadimenol, toluene, and p-xylene) and many psychoactive control agents increase motor activity. It has been suggested that hyperactivity may be evidence of a specific effect on the nervous system (Crofton et al. 1988).

Neuromuscular Sum
Acrylamide



2-Hydroxyethyl Acrylate

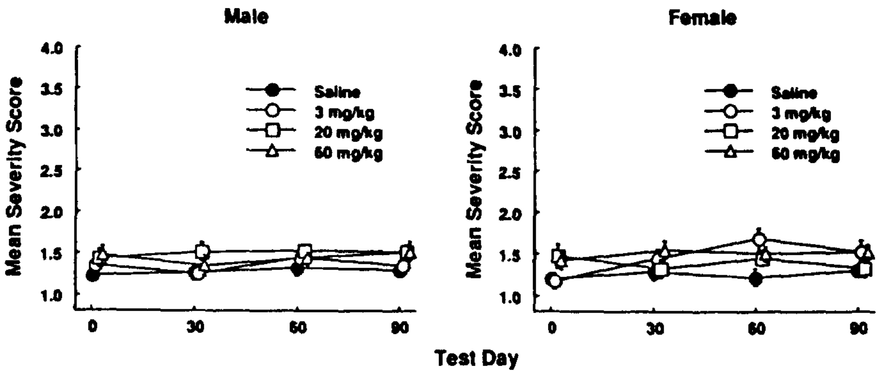


FIGURE 1. *Average severity scores ±S.E. for the neuromuscular domain for male and female rats during exposure to various doses of acrylamide (top) or 2-HEA (bottom). Data for each animal's response on tests making up the neuromuscular domain were normalized on a 1 to 4 scale, and the cumulative score was divided by the number of tests (six) making up the neuromuscular domain. Data are average domain scores +/- for 10 rats per group.*

SOURCE: Adapted from Moser 1991

A recent interlaboratory comparison (Crofton et al. 1991) found a high degree of comparability in the data generated by different devices and experimental protocols, including within laboratory control variability, within laboratory replicability of control data, between laboratory variability in the effects of prototypic chemicals, and between laboratory comparison in the control rates of habituation. In the interlaboratory comparison, effects of several psychoactive or neurotoxic agents were studied in five different testing apparatuses, including a figure-eight maze, Woodard photoactometer, Motron photoactometer, square field with photocells, and rack-mounted cages with photocells, Studies were performed under the conditions standard for each laboratory using either Long-Evans or Fischer-344 rats. Figure 2 shows the effects of triadimefon, a fungicide, on motor activity measured in six different laboratories. All laboratories reported that triadimefon increased motor activity, with a peak effect observed at about 100 mg/kg. Doses higher than 100 mg/kg had a less stimulatory effect on motor activity. Other chemicals studied include

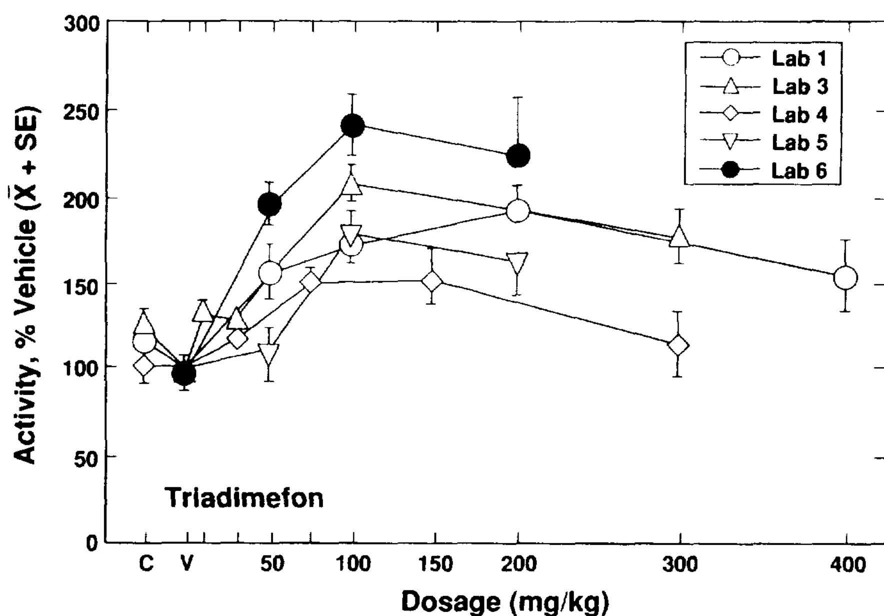


FIGURE 2. *Dose-related effects of triadimefon on automated motor activity measured in five different laboratories. Data are plotted as the percentage of the vehicle control mean for each laboratory. Control means ranged from 257 to 4,033 activity counts per session (25 to 90 minutes).*

SOURCE: Adapted from Crofton et al. 1991

d-amphetamine, scopolamine, carbaryl, chlorpromazine, endosulfan, physostigmine, cypermethrin, and methylscopolamine. Similar agent-induced effects on motor activity were observed by the different laboratories.

SPECIALIZED TESTS OF NEUROBEHAVIORAL FUNCTION

Motor Dysfunction

Anger (1984) reviewed the effects of 588 chemicals for which the American Conference of Governmental Hygienists (ACGH) had recommended threshold limit values (TLV) and found that 167 (28 percent) had a TLV based totally or in part on neurological or behavioral effects. Anger also evaluated the types of neurotoxic effects reported for the 167 chemicals and reported a list of 69 different effects, which were classified under 5 major groups, including motor, sensory, cognitive, general changes, and affective/personality. Of the 69 different effects, 20 percent (14) were motor deficits. The 14 different types of motor effects can be grouped into 4 categories, including tremor, convulsions and spasms, weakness, and incoordination (Newland 1988). Examples of chemicals known to produce motor dysfunction in humans are acrylamide, 2,5-hexanedione, carbaryl, chlordecone, 1 -methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and manganese. Table 4 summarizes tests developed to assess motor dysfunction in animal neurobehavioral pharmacology and toxicology. Because tremors and weakness in animals have **a** high frequency of occurrence, procedures used to measure them will be discussed in greater detail.

Tremor. Chemical-induced tremor is frequently reported following exposure to neurotoxicants. Anger and Johnson (1985) reviewed 750 chemicals affecting the nervous system and identified 120 nervous system-related effects. Anger (1990) listed the behavioral effects produced by 25 or more of the 750 chemicals and found tremor/twitching to be associated with 177 agents.

Various techniques and approaches have been devised to measure tremor in animals, and these have been reviewed critically by Newland (1988). One procedure used to study tremor in rats is that developed by Gerhart and colleagues (1982)) who assessed whole body movement by measuring the displacement of a freely moving platform attached to a load cell-transducer. A spectral analyzer was used to quantitate chemical-induced alternations in movement. Figure 3 shows dose- and time-related tremorigenic activity produced by p,p'-DDT, an organochlorine insecticide (Hudson et al. 1985). In this study, rats were given 25, 50, 75, or 100 mg p,p'-DDT/kg and tested 12 hours later or given 75 mg/kg and tested 2, 5, 12, or 24 hours later. p,p'-DDT increased the average power of whole body movements in a dose-dependent manner, with the greatest increases occurring in the 7.5 to 15.0 Hz regions of the spectrum. The peak time of effect for p,p'-DDT was 5 to 12 hours

TABLE 4. *Examples of behavioral procedures to measure motor dysfunction in animals*

Function	Test	Reference
Tremor	Rating scale	Hong et al. 1984
	Spectral analysis	Newland 1988
		Gerhart et al. 1982
Convulsions	Rating scale	Racine 1972
Weakness	Grip strength	Pryor et al. 1983a
	Suspension from rod	Molinengo and Orsetti 1976
	Swimming endurance	Bhagat and Wheeler 1973
	Response force titration	Elsner et al. 1988
	Nautilus	Newland 1988
Incoordination and abnormal movements or posture	Rotorod performance	Bogo et al. 1981
	Rope climbing	Carlini et al. 1967
	Inclined screen	Graham et al. 1957
	Hindlimb splay	Edwards and Parker 1977
	Automated hindlimb test	Tanger et al. 1984
	Gait analysis	Lee and Peters 1976
	Negative geotaxis	Pryor et al. 1983a

postdosing. The apparatus described by Gerhart and colleagues (1982) has been used to study the biological substrate underlying tremor produced by chlordecone, p,p'-DDT, and permethrin (Tilson et al. 1985, 1966).

Weakness. In evaluating the most frequently reported behavioral and neurological effects produced by 750 chemicals affecting the nervous system, Anger (1990) found 179 agents produced weakness. One technique used in many laboratories to measure chemical-induced weakness in rodents is the forelimb and hindlimb grip strength procedure, which measures the force required to cause an animal to release its grip from a bar or wire screen (Meyer et al. 1979; Haggerty 1989; Kulig 1989; Moser 1989; O'Donoghue 1969). Kulig (1989) dosed rats with 3.6, 7.2, or 14.4 mg acrylamide/kg for 12 weeks and measured grip strength every 3 weeks during dosing and for up to 6 weeks after cessation of dosing. Figure 4 shows that the higher dose of acrylamide significantly decreased grip strength after 9 weeks of dosing. Strength began to recover following cessation of dosing. Grip strength measurements are included in many neurotoxicity testing batteries (Haggerty 1989; Kulig 1989; Moser 1989; O'Donoghue 1989; Tilson 1990*b).*

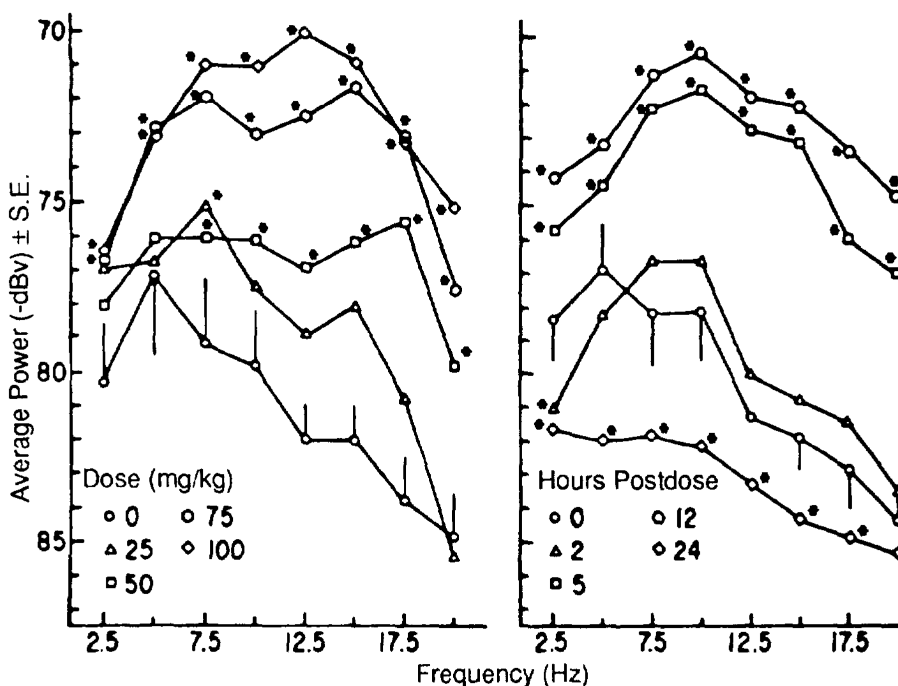


FIGURE 3. *Dose- and time-related effects of p,p'-DDT on whole body movement of rats. Five hours after oral dosing with 25 to 100 mg/kg p,p'-DOT, whole body movements were evaluated by spectral analysis (left). A separate experiment examined the effects of 75 mg/kg p,p'-DDT at 2 to 24 hours after dosing (right). Data are average values (-dBV)=± S.E. for six rats per group.*

SOURCE: Data derived from Hudson et al. 1985

Sensory Dysfunction

Many chemicals are known to affect sensory systems. Of the 69 separate neurotoxic effects reported in ACGH documentation of TLV determinations for chemicals used occupationally, 10 (14 percent) concerned effects on sensory systems (Anger 1984). In his review of the effects produced by 750 agents having neurotoxicity, Anger (1990) reported visual and equilibrium disorders were associated with 121 and 135 chemicals, respectively. Tactile, pain, and auditory disorders were associated with 77, 64, and 37 chemicals, respectively. Examples of chemicals having effects on sensory systems include toluene (auditory), methyl mercury (visual), acrylamide (somatosensory), parathion (pain), and 3-methylindole (olfaction).

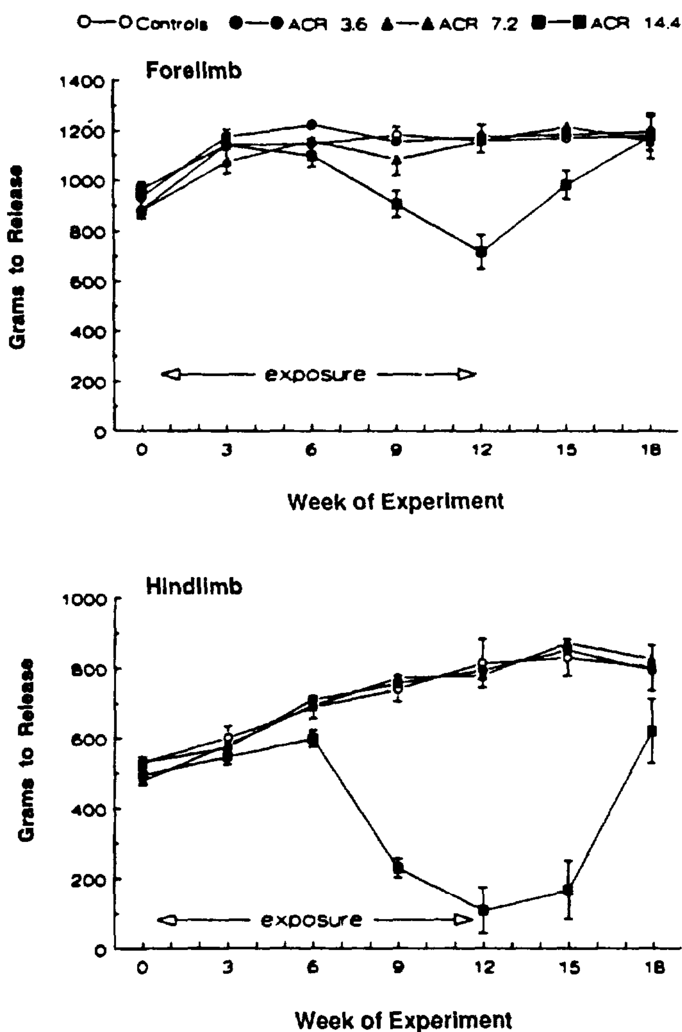


FIGURE 4. *The effects of subchronic exposure to acrylamide on forelimb and hindlimb grip strength of rats. Animals were dosed 5 days per week (intraperitoneal) with 3.6, 7.2, or 14.4 mg/kg acrylamide. Measurements were made prior to dosing; 3, 6, 9, or 12 weeks during dosing; and 3 and 6 weeks after cessation of dosing. Data are average scores of eight rats per group.*

SOURCE: Reprinted with permission from Kulig, B.M. A neurofunctional test battery for evaluating the effects on long-term exposure to chemicals. *J Am Coll Toxicol* 8(1):71-83, 1989. Copyright 1989 by Mary Ann Liebert, Inc. (New York City).

Several tests developed to assess animals for chemical-induced neurotoxicity can be classified into three categories: classical conditioning, operant behavior, and reflex modification (Maurissen 1988). Neurobehavioral procedures used to assess sensory function are based on psychophysical methodology, which is the study of the relationship between a physical dimension of a stimulus and the response that is generated (Maurissen 1988). Psychophysical assessments involve the presentation of a stimulus along some physical dimension (wave length, brightness) and require the animal to make or withhold a specific response in the presence or absence of that stimulus. Psychophysical methods have been recently described by Maurissen (1988) and include the method of limits, constant stimuli, adjustment, and tracking. Table 5 summarizes various tests that have been used to assess sensory function in animals. Two representative procedures that have been used to measure chemical-induced changes in sensory function are described below.

Multisensory Conditioned Avoidance Response (CAR). Pryor and colleagues (1983b) used a tone-intensity discrimination task in rats to determine the ototoxic effects of toluene exposure: they exposed weanling rats to 1,200 or 1,400 ppm toluene for 14 hours a day, 7 days per week, for 5 weeks, Some animals were trained on a multisensory CAR task during the last week of exposure or during the first or third weeks following cessation of exposure. Training consisted of presentation of one of three stimuli (i.e., light, tone, or nonaversive current on the grid floor of the test chamber) prior to the presentation of an aversive electric shock. The rats could avoid presentation of the aversive shock by pulling or climbing a pole suspended from the ceiling of the test apparatus during the

TABLE 5. *Examples of behavioral tests used to assess sensory dysfunction in animals*

Test Type	Procedure	Reference
Classical conditioning	Conditioned avoidance	Pryor et al. 1983*b*
Operant behavior	Conditioned suppression	Fox et al. 1982
	Tracking procedure	Maurissen et al. 1983
	Flicker sensitivity	Merigan 1979
	Constant stimulus	Stebbins and Moody 1979
	Discrimination response	Hastings 1990
	Duration discrimination	Daniel and Evans 1985
Reflex modification	Prepulse inhibition	Eastman et al. 1987
		Crofton et al. 1990
		Fechter and Carlisle 1990

presentation of the warning stimuli. Rats learned to avoid the aversive shock whenever the nonaversive shock or light warning stimuli were presented, but not when the 20 kHz auditory stimulus was presented (figure 5). In a subsequent manipulation beginning 2.5 months after the last exposure, the rats were given a series of training sessions that varied the frequency (i.e., 4, 8, 12, 16, or 20 kHz) and intensity (20 to 60 dB) of the auditory warning stimuli. The hearing of the toluene-exposed rats was unimpaired at 4 kHz, slightly impaired at 8kHz, and markedly impaired at 16 and 20 kHz. Subsequent experiments (Rebert et al. 1983) found auditory dysfunction in these animals using brain stem-evoked potential methodology to determine auditory thresholds.

Reflex Modification. In the prepulse inhibition procedure, a weak stimulus presented before a second eliciting stimulus can suppress a reflex response to the eliciting stimulus. Because the degree of suppression is dependent on the intensity of the inhibiting stimulus, sensory functioning can be assessed by determining the intensity of the inhibiting stimulus required to produce significant inhibition of the reflex (i.e., a sensory threshold). Crofton and coworkers (1990) gave rats a single exposure to TMT (3, 5, or 7 mg/kg), and low (5 kHz) and high (40 kHz) auditory function was assessed 11 weeks later. TMT produced a dose-dependent decrease in the amplitude of the acoustic startle reflex (figure 6). However, TMT increased the auditory threshold at 40 kHz only. Morphological examination of the inner ear confirmed a preferential loss of outer hair cells, which are associated with high-frequency hearing.

Cognitive (Learning and Memory) Dysfunction

Of the 69 signs and symptoms in humans exposed to neurotoxicants identified by Anger (1984), only about 6 percent were classified as cognitive. Memory problems were associated with 33 of 750 chemicals having neurotoxic effects; examples of chemicals that affect memory in humans include carbon disulfide, styrene, organ ophosphates, lead, and mercury (Anger 1990). The concern about cognitive dysfunction is greater in the area of developmental neurotoxicology where there is a frequent association between mental retardation and congenital anomalies (Illingworth 1959; Smith and Bostian 1964).

The experimental assessment of cognitive functions such as learning and memory in animals is confounded by the fact that such processes must be inferred from a change in behavior following exposure. Learning is the process of adaptation to changed contingencies, whereas memory is a construct used to describe the influence of previous events on behavior. Furthermore, alterations in learning and memory must be separated from other changes in performance that depend on intact sensory and motor functions and the motivational state of the animal. Toxicant-induced changes in learning and memory should be demonstrated over a range of doses and neurobehavioral procedures.

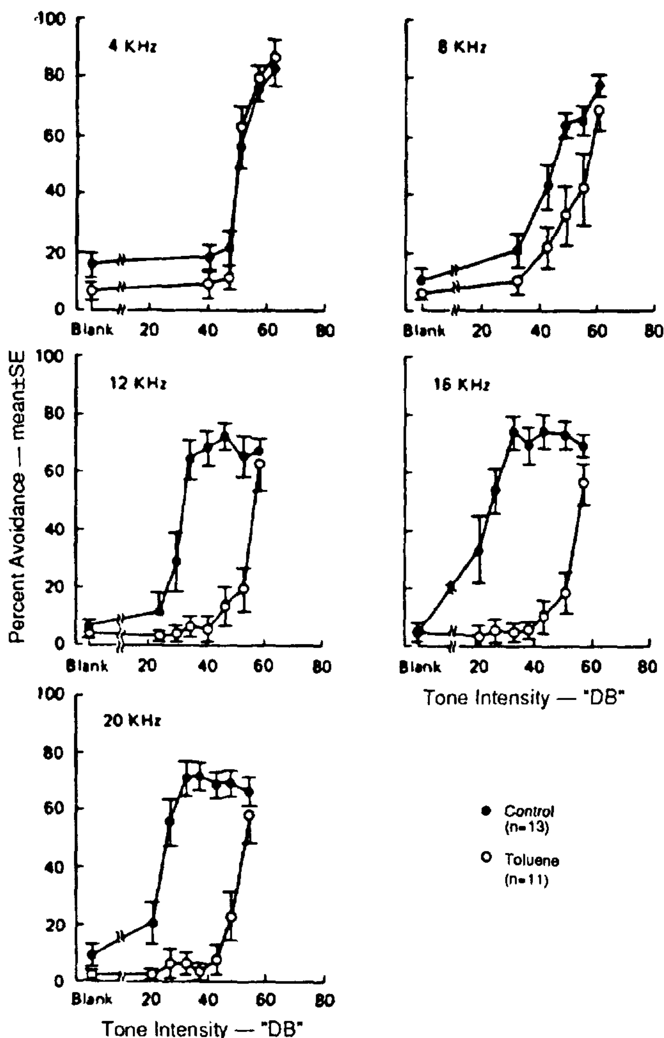


FIGURE 5. *Effect of subacute exposure to toluene on performance of a conditioned avoidance response to tone as a function of the frequency and intensity of the stimulus. Testing began approximately 2.5 months after the last exposure.*

SOURCE: Reprinted with permission from Pryor, G.; Dickinson, J.; Howd, R.A.; and Rebert, C.S. Transient cognitive deficits and high-frequency hearing loss in weanling rats exposed to toluene. *Neurobehav Toxicol Teratol* 5:53-57, 1983*b*. Copyright 1983 by Pergamon Press, plc. (Elmsford, NY).

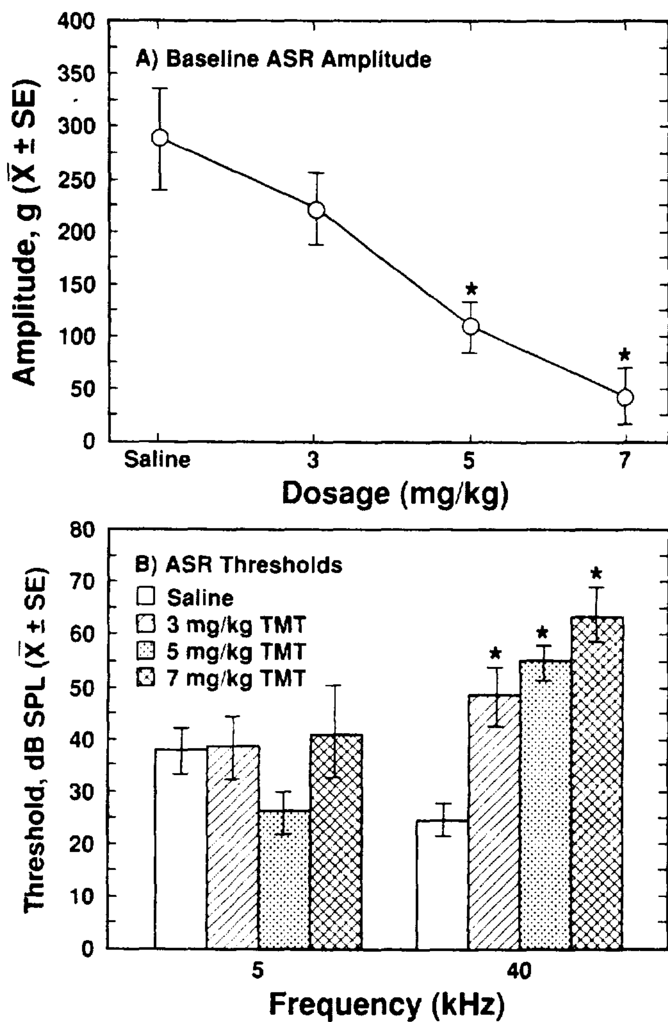


FIGURE 6. *TMT-induced alterations in auditory thresholds and the acoustic startle reflex amplitude. Panel A: TMT produced a significant depression in baseline startle response. Data are group averages ±S.E. of the reflex on blank* trials. *Panel B: TMT-induced increases* in *frequency-dependent auditory* thresholds. Data are group *averages ±S.E. of the auditory thresholds for 5- and 40-kHz pure tones. Asterisks indicate a significant difference from saline control group (p<0.025).*

SOURCE: Crofton et al. 1990. Copyright 1990 by Academic Press (Orlando, FL).

Neurobehavioral techniques to assess learning and memory have been reviewed by Peele and Vincent (1989) and Heise (1984). In general, tests for learning and memory can be categorized into measures of nonassociative learning, classical conditioning, and instrumental procedures (Heise 1984). Table 6 summarizes several techniques that have been used to assess neurotoxicant-induced changes in learning and memory in animals. Examples of three procedures that have been used to assess learning and memory in rats are described in greater detail below.

Long-Delay Flavor Aversions. The conditioned flavor aversion procedure is a classical conditioned task based on the finding that rats will avoid consuming solutions with flavors previously paired with the consequences of an aversive stimulus such as an injection of lithium chloride. If delays are imposed between the initial intake of the flavor and injection of lithium, the aversion to the flavor is weaker. Peele and colleagues (1989) exposed rats with TMT and tested them 30 days later for conditioned flavor aversions. Lithium was injected 0.5, 3, or 6 hours after rats were allowed to drink an 0.1 percent w/v sodium saccharin solution. Two days later the rats received concurrent access to water and saccharin and the preference for the saccharin solution was determined.

TABLE 6. *Behavioral endpoints used to assess learning and memory in animals*

Type of Function	Procedure	Reference
Habituation	Startle reflex	Overstreet 1977
Classical conditioning	Eye-blink reflex	Yokel 1983
	Flavor aversion	Riley and Tuck 1985; Peele et al. 1989
Instrumental conditioning	Passive avoidance	Walsh et al. 1982a
	Active avoidance	Tilson et al. 1987
	Radial arm maze	Walsh et al. 1982b
	Biel water maze	Vergieva and Zaikov 1981
	Morris water maze	Tilson et al. 1990
	Repeated acquisition	Paule and McMillan 1986
	Delayed alternation	Bushnell 1988; Rice and Karpinski 1988
	Matched to position	Bushnell et al. 1991
	Reversal learning	Rice 1990

Figure 7 shows that rats injected with TMT 0.5 or 3 hours after pairing with saccharin were not different from controls. However, rats in the 6-hour delay group showed a significant impairment relative to controls. Subsequent experiments found similar deficits in TMT-exposed rats in tasks for somatosensory and gustatory avoidance learning. TMT also produced a relatively specific destruction of pyramidal cells in the hippocampus, an area important for short-term memory.

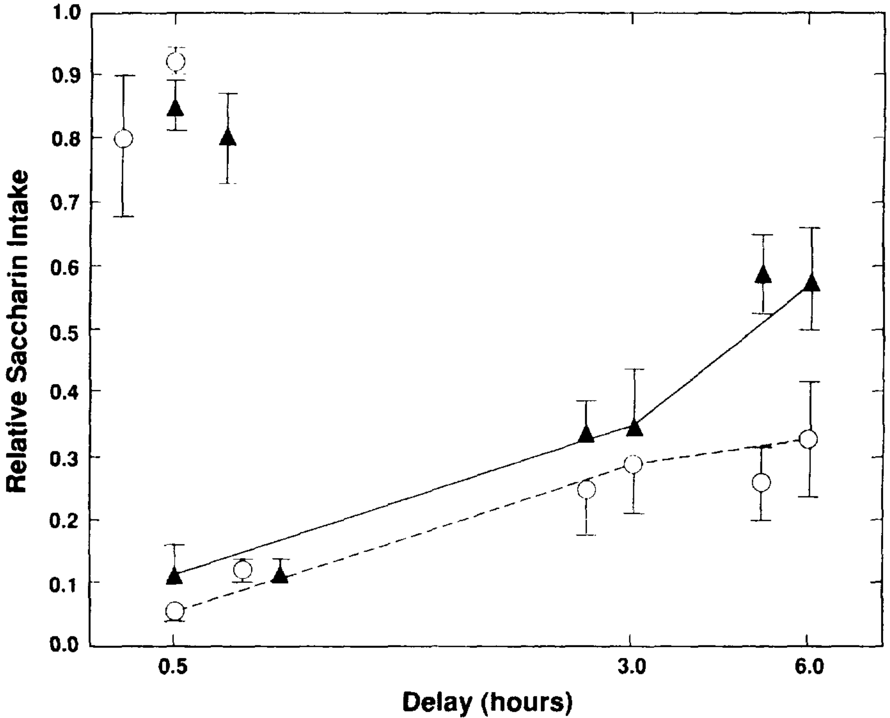


FIGURE 7. *Relative saccharin intake (saccharin intake expressed as a proportion of total fluid intake) for rats receiving lithium chloride following saccharin consumption during a flavor preference test. Rats were given either 8 mg/kg TMT (triangles) or saline (circles) and tested 30 days later. Data are average preference scores ± S.E. of nine rats as a function of the delay separating saccharin availability and lithium chloride administration.*

SOURCE: Adapted from Peele et al. 1989. Copyright 1989 by Springer-Verlag (New York).

Morris Water Maze. Spatial memory tasks are being used with greater frequency in behavioral toxicology. One such task is the Morris water maze, which measures the ability of a rat to find a submerged platform located in a vat of water (Morris et al. 1986). Tilson and coworkers (1990) gave rats a single dose of tris (2-chloroethyl) phosphate (TRCP) and tested for learning and memory capabilities 3 weeks later. When rats were trained to swim to a platform always hidden in the same location in the water maze, a significant attenuation in the rate of acquisition was observed (figure 8). When TRCP-treated rats were trained in a task where the position of the maze changed daily, TRCP-exposed rats showed clear deficits in acquisition, Examination of the brains of the TRCP-treated animals revealed significant destruction of pyramidal cells in the CA3 and CA4 regions and of granule cells in the dentate gyrus of the hippocampus. Other experiments (Tilson et al. 1990) found that the neurobehavioral deficits produced by TRCP could be blocked by prior administration of atropine or chlordiazepoxide, both of which reduced the severity of TRCP-induced seizures.

Discrete-Trial Operant Procedure. Working, short-term memory can be evaluated by using a delayed response task with a spatial location as the discriminative stimulus. Bushnell (1988) trained rats to receive food for pressing one of two retractable levers in the choice phase of a trial if that lever had been presented in the prior sample phase of that trial. Accuracy of the choice decreases whenever delays are interposed between the sample trial and the choice, with the largest effect occurring with the longest delay. Choice accuracy also decreases more at long delays between sample and choice with shorter times between trials, indicating that interference from previous trials impairs memory but not discrimination. TMT (7 mg/kg, intravenous [IV]) was given to rats trained to perform on the spatial delayed response memory task, and behavior was tested up to 4 weeks later. TMT significantly altered the retention gradient 4 weeks after treatment (figure 9), suggesting a disruption of memory associated with temporal delays. Neurochemical studies performed at the end of the behavioral experiments found a highly significant correlation between deficits in spatial delayed responding and levels of glial fibrillary acidic protein (GFAP) in the hippocampus. GFAP levels in the CNS increase over basal levels in response to cellular injury caused by several known neurotoxicants (O'Callaghan 1988).

Schedule-Controlled Behavior

Schedule-controlled behavior refers to a special application of operant behavior, which can be defined as movement that operates on or alters the environment. As described in a previous section, operant responses become conditioned or learned when their frequency is modified by the consequences of the response through a process known as reinforcement. Schedule-controlled behavior is based on the principle that reinforcement is usually applied intermittently and that behavior can be predicted on the basis of the programing or scheduling of

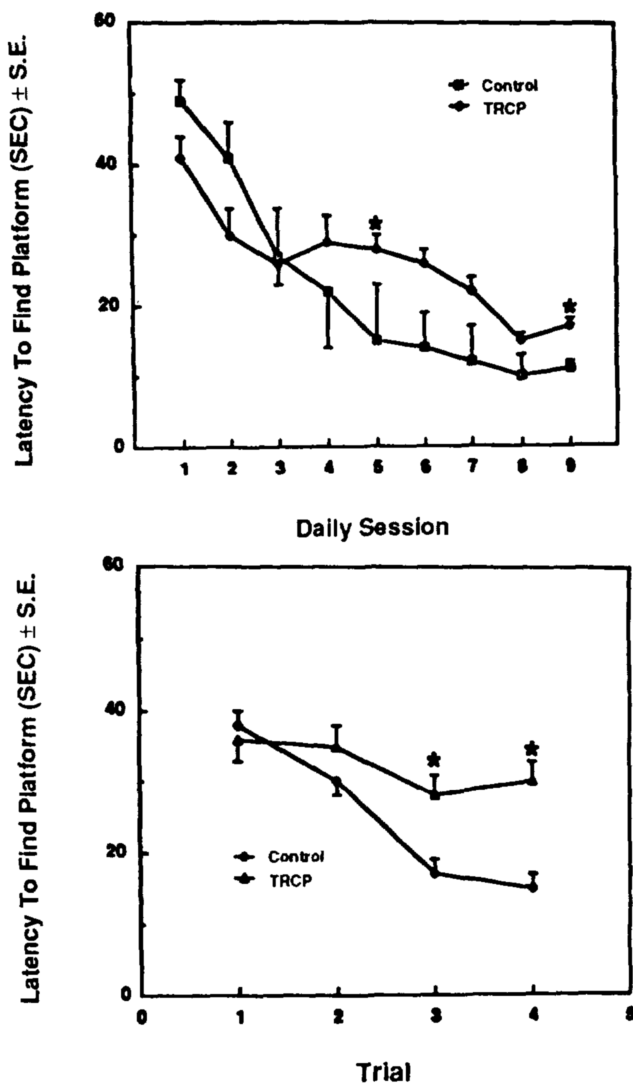


FIGURE 8. *Effects of TRCP exposure on acquisition of spatial memory task in rats. (Top) Animals were required to find submerged platform located in the same place each day (reference memory task) for four trials per day for 9 days. (Bottom) Location of submerged platform changed each day (repeated acquisition task). Data are average latencies to find platform ±S. E. Asterisks indicate significant difference from control group (p<0.05).*

SOURCE: Tilson et al. 1990. Copyright 1990 by Academic Press (Orlando, FL).

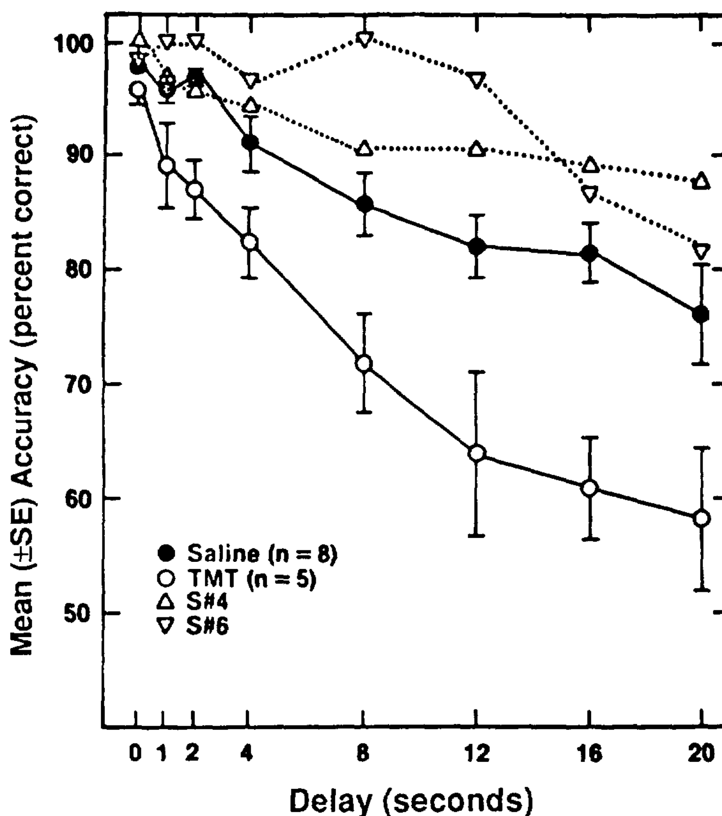


FIGURE 9. *Effect of TMT, 7 mg/kg IV on retention gradient during week 4 after exposure. Values are averages ±S. E. for controls* (●, n=8) and *TMT-exposed* rats (O, n=5).

SOURCE: Adapted with permission from Bushnell, P.J. Effects of delay, inter-trial interval, delay behavior and trimethyltin on spatial delayed response in rats. *Neurotoxicol Teratol* 10:237-244, 1988. Copyright 1988 by Pergamon Press, plc. (Elmsford, NY).

reinforcement that follows responding, For example, reinforcement can occur after a certain number of responses have been emitted, after a specified period of time, or after a combination of both. A schedule of reinforcement specifies the relation between a specific response and the delivery of reinforcement. For example, fixed interval (FI) schedules require that a specific period of time elapse before a response is reinforced, whereas a fixed ratio schedule requires that a certain number of responses occur before reinforcement is delivered. Interval and ratio schedules can also be variable where the time or number of

responses varies around some average. A complete description of simple, compound, complex, and higher order schedules can be found elsewhere (Ferster and Skinner 1957).

Schedule-controlled behavior reflects the net integrated sensory, motor, and cognitive abilities of an organism. The study of neurotoxicants on human schedule-controlled behavior is limited, although it has been used in psychopharmacology to study the effects of psychoactive drugs. Schedule-controlled behavior has been used extensively in animal behavioral toxicology where it has been shown to be sensitive to a wide range of neurotoxicant agents (Tilson 1987; Rice 1988). Schedule-controlled behavior is useful in that the experimental animal frequently serves as its own control. Two examples in which schedule-controlled behavior has been used to study neurotoxicant effects on behavior are described below.

Effects of Lead on Intermittent Schedules of Reinforcement. Several studies have demonstrated that developmental exposure to lead has significant effects on operant behavior tested at later ages (Rice et al. 1979; Cory-Slechta and Thompson 1979). Cory-Slechta and colleagues (1985) exposed male weanling rats repeatedly to 25 ppm lead acetate via the drinking water. The rats were trained to respond for food on an FI 1 -minute schedule of reinforcement starting at 50 days of age. Performance was assessed over 90 experimental sessions or 136 days. Figure 10 shows that lead exposure resulted in an increased response rate during the first 40 sessions. However, after 40 sessions, response rates of the lead-exposed group approached those of the control group. These data are important in that behavioral effects were observed in animals having blood lead levels ranging from 15 to 20 upg/dL throughout the study. Such blood levels are within the range observed in many children (Mahaffey et al. 1982).

Effects of Solvents on Schedule-Controlled Behavior. Solvents include several types of chemicals (i.e., aromatic, aliphatic, and halogenated hydrocarbons: alcohols: esters; ketones; aliphatic nitrites; anesthetics: and propellants). These volatile organic solvents are prevalent in the environment, especially in the air and drinking water. The behavioral effects of the volatile solvents have been studied by many investigators; however, comparisons between studies have been confounded due to differences in behavioral technique, routes of exposure, and lack of adequate dose-response determinations. Glowa (1985) compared the range of effects of several volatile organic solvents using schedule-controlled behavior in mice. Response on an FI 60-second schedule of reinforcement was studied during exposure to the solvents. Figure 11 shows that with few exceptions (e.g., toluene) solvents from several different chemical classes produced dose-dependent decreases in the rate of responding. Glowa (1985) suggested that the behavioral toxicity of the solvents should be described as a decrease in response rate. Decrements in responding were more closely associated with higher

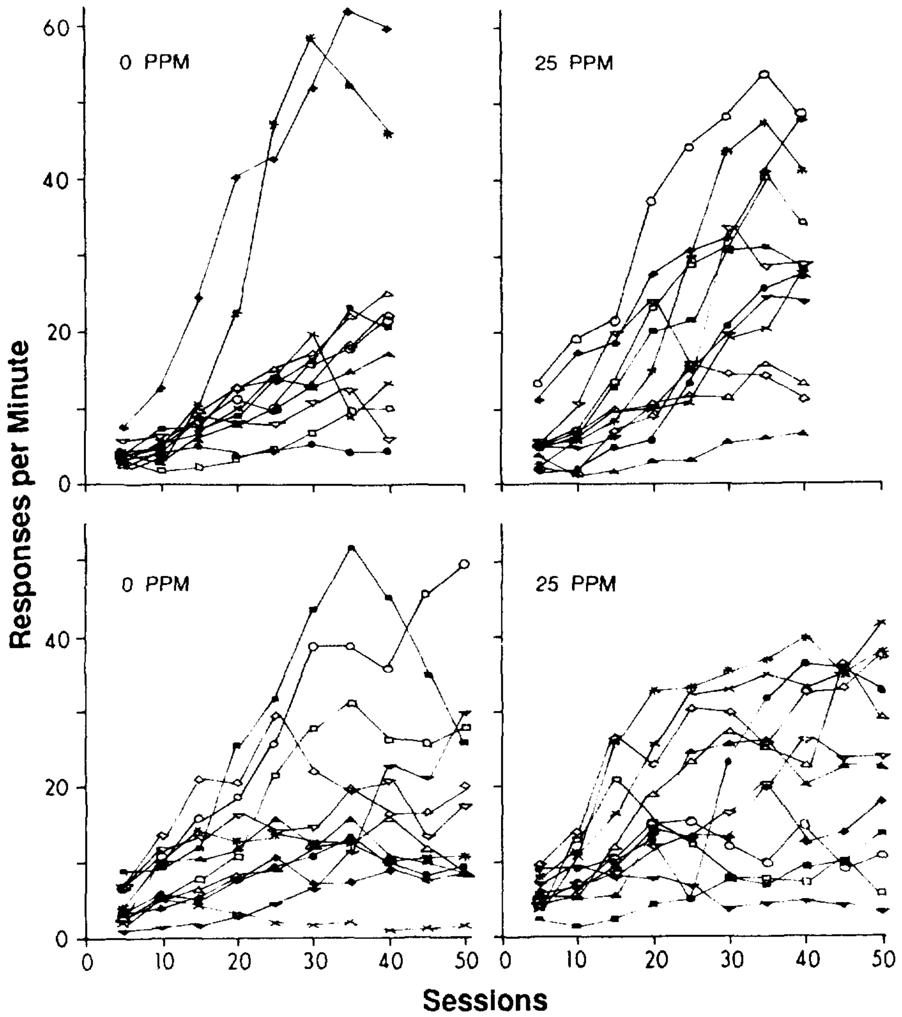


FIGURE 10. *Individual overall response rates (responses per minute) of rats (n= 12 per group) exposed from weaning to 25 ppm sodium acetate (controls, top panels) or 25 ppm lead acetate (bottom panels) over 40 sessions of an FI 1-minute schedule of reinforcement. Increased response rates of the lead-exposed rats were determined using a randomization test using the linear coefficients resulting from the cubic polynominal fit to each animal's curve.*

SOURCE: Cory-Slechta et al. 1985. Copyright 1985 by Academic Press (Orlando, FL).

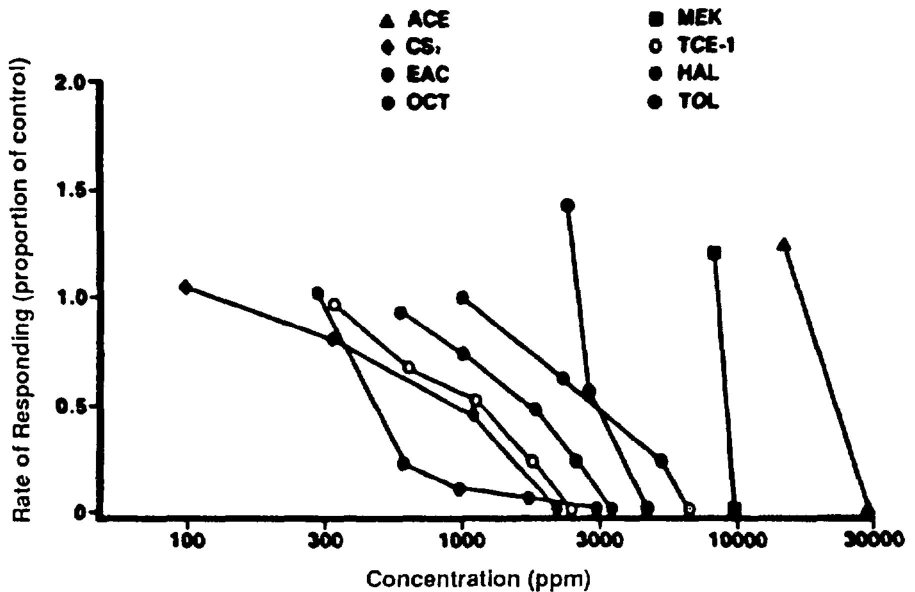


FIGURE 11. *Average rates of responding (proportion of control) as a function of exposure concentration (ppm) for the rate-decreasing effects of cumulative exposure to acetone (ACE), carbon disulfide (CS_2), ethyl acetate (EAC), n-octane, (OCT), methyl ethyl ketone (MEK), trichloroethane (TCE- l), halothane (HAL), and toluene (TOL), for FI 60-second responding in mice.*

SOURCE: Glowa, J.R. Behavioral effects of volatile organic solvents, In: Seiden, L.S., and Balster, R.L., eds. *Behavioral Pharmacology: The Current Status.* New York: Alan R. Liss, 1985. pp. 537-552. Copyright © 1985 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

concentrations and were relatively linear. This latter aspect is important because it permits direct comparison of EC_{50} values for diverse classes of chemicals. Linear dose-response functions are also useful in the determination of benchmark doses or no-observed-adverse-effect or lowest-observed-adverse-effect levels used in quantitative risk assessment.

SUMMARY

The use of neurobehavioral techniques in toxicology has increased dramatically over the past several years. Several national and international groups have recommended that neurobehavioral tests be included in the initial stages of

hazard identification, and regulatory agencies have responded by preparing testing guidelines or requiring behavioral test data for premarket approval of environmental and pharmaceutical chemicals. In addition, neurobehavioral data have been used to set exposure limits in the workplace.

In the future, neurobehavioral data will be used more frequently in the area of risk assessment, which has been defined as the "characterization of the potential adverse effects of human exposure to environmental hazards" (National Academy of Sciences 1983). Good risk assessment depends on the ability to determine whether a particular agent is or is not causally linked to a particular health effect and on the availability of dose-response data for quantitative risk assessment. Neurobehavioral techniques used in animal behavioral toxicology measure neurobiological functions similar to those measured in humans. In addition, neurobehavioral procedures can be used in longitudinal studies where the onset and duration of effects of chemical exposure can be measured in the same animal. Neurobehavioral techniques are also amenable to the study of tolerance and compensation following repeated exposure or following recovery of function that can occur following cessation of exposure. Therefore, neurobehavioral procedures provide a valuable tool for research designed to reduce major uncertainties associated with the risk assessment process, such as animal to human extrapolation (homology of animal models) and dosing issues (i.e., high-to-low dose, acute vs. repeated dosing, and continuous vs. episodic dosing).

Although the use of neurobehavioral procedures has had a significant impact on neurotoxicology, their use in the risk assessment process and in monitoring populations for possible subtle changes in neurobiological function will be limited if additional research is not done to understand the neural substrates underlying neurobehavioral endpoints. The ability to link chemically induced behavioral changes to alterations at the neurophysiological, neurochemical, and neuroanatomical levels will lead to a greater acceptance of the validity and reliability of neurobehavioral endpoints in defining adverse effects of chemicals on the nervous system.

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Discussion

Q: Do you have any simple tests for physical dependence that will give evidence of neurotoxicity?

A: I think some of the measurements in the functional observational battery exam might be able to show withdrawal signs due to morphine because many of these tests are designed to detect chemical-induced changes in nervous system excitability or arousal. Under the appropriate conditions, schedule-controlled behavior has been used to determine withdrawal.

Q: It is known that amphetamine addicts eat an awful lot and sleep a long time after they crash. The thing that we have to show is that they actually eat more or sleep longer-that would somehow be evidence of physical dependence.

A: There are behavioral tests involving diurnal measures of consummatory responses (i.e., eating and drinking behaviors) and activity patterns that could be used to study consequences of withdrawal from physical dependence.

Q (Ricaurte): If you take as a positive control a compound like MPTP (the dopaminergic neurotoxicant) and you put it through a screen such as the screen you have described in the rat, what kind of information does the screen give?

A: The rat is not very sensitive to MPTP, so a neurological screen would probably not detect the presence of neurotoxicity. If a chemical produces motor dysfunction in a test species, then a neurological screen such as the functional observational battery should detect it.

Q (Ricaurte): A related question is to what extent is consideration given not only to rodents but also to using a nonhuman primate to screen.

A: For premarket approval of pesticides and for toxic substances, EPA requires testing in rodent species. In certain cases, avian species are required for assessment of organophosphate-induced delayed neuropathy.

Q (Ricaurte): Is there a particular sequence or array of tests that you use to cover the eventuality that one animal may not be adequate?

A: Our testing guidelines do not specify a strain of rats. In the case of the developmental neurotoxicity, there is one strain of rats that is discouraged. Multispecies assessments are important to cover this eventuality.

Q (Sobotka): Just one clarification. It was my understanding that with MPTP [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine] you can get effects in rats at proper doses. It seems that there is a growing misconception that a rat is not

responsive to MPTP. It is true that rats do not reproduce the lasting neuropathological effects seen in primates, dogs, and humans, but then you're talking about the FOB identifying the chemical. A rat is able to do that. Where you may have a problem is when you do the dose-response characterization stage where you're looking for a lowest-effect level or no-effect level. Then you have a problem.

COMMENT (Ricaurte): Is the endpoint that you are using specific? Is it a measure of, for example, dopamine levels?

A (Sobotka): No, no. I'm talking about an observational battery. I have not done this myself; this is based on the literature. The only animal species that I have used MPTP on is the dog. And we can see these types of neurological problems in the dog. In the rodent relative to the primate, you have to use higher doses, but according to the literature, the kinds of effects that you will see here would be picked up by FOB.

COMMENT (Tilson): Jim O'Callaghan may have something of an answer for this, because he has looked at rats.

A (O'Callaghan): I'm going to defer to my colleague, Diane Miller, for behavioral expertise.

A (Miller): In the literature, you can pick up catalepsy in both mice and rats if you go to high enough doses. So with the FOB, you would pick that up. Now, that is not a long-lasting effect. You have the question of reversibility and whether an effect is related to any kind of substantia nigra damage, any kind of dopaminergic long-lasting changes.

A (Tilson): I think that the combined use of a functional observational battery, motor activity, neuropathological assessment, and GFAP measurements would pick up most neurotoxicants as we know them today. Lew?

Q (Seiden): The time after the insult has to be an important variable in assessing the toxicity vs. the pharmacology of these compounds or drugs. I don't know of a good benchmark for discriminating between the short effects and the more long-lasting, perhaps permanent effects of these drugs. Do you have any, as a regulatory agency?

A (Tilson): Changes lasting more than 24 to 48 hours after dosing cause a greater level of concern than those that are acutely reversible.

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Behavioral Consequences of Partial Monoamine Depletion in the CNS After Methamphetamine-Like Drugs: The Conflict Between Pharmacology and Toxicology

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INTRODUCTION

There is considerable evidence that dopamine (DA) and serotonin (5-hydroxytryptamine [5-HT]), by virtue of their role as tonic neurotransmitters in the central nervous system (CNS), play an important role in locomotion and motor control, food intake, mood, sexual behavior, aggression, sleep, and pain perception (Seiden et al. 1979; Carlsson 1988; Iversen et al. 1990; Seiden and Dykstra 1977). Part of that evidence is derived from pharmacological studies: Drugs that either directly or indirectly stimulate DA receptors have a characteristic profile of behavioral effects (Creese and Iversen 1973, 1975; Goodman and Gilman 1990). For example, amphetamine (AMPH) is an indirect DA agonist. At low-to-moderate doses, AMPH increases locomotion, apparently involving DA in the limbic forebrain; decreases food and water intake; has rate-dependent effects on schedule-controlled behavior; and functions as a positive reinforcer. At higher doses, AMPH induces species-specific stereotypic behaviors involving striatal DA (Cooper et al. 1991; Ellinwood 1974; Kelleher and Morse 1964; Scheel-Kruger and Randrup 1967). In humans, AMPH increases positive mood, has a high abuse liability, and when used in high doses, can induce stereotypic and maladaptive behaviors. When AMPH is used repeatedly, paranoid psychosis develops that may also be related to a hyperdopaminergic state. AMPH can also prevent sleep and is prescribed in the treatment of narcolepsy (Goodman and Gilman 1990). Although some of the effects of AMPH may involve 5-HT and/or the norepinephrine (NE) mechanisms, DA appears to play a particularly prominent role (Ando et al. 1985; Azzaro et al. 1974; Chiueh and Moore 1975; Heikkila et al. 1975; Raiteri et al. 1979). It also should be noted in this context that DA antagonists are used as antipsychotic agents and DA agonists are used to treat Parkinson's disease.

Similarly, pharmacological studies suggest that 5-HT plays an important role in the behavioral effects of antidepressant, antipsychotic, and antianxiety drugs (Asberg and Wagner 1986; Garattini and Samanin 1988; Marek et al. 1988; Peroutka and Snyder 1980; Plic 1987; Seiden and O'Donnell 1985; Sulser 1983 *a;* Vetulani et al. 1976). Several clinically efficacious antidepressant drugs block the 5-HT transporter (e.g., chlorimpramine, imipramine, fluoxetine) (Cowen 1990; Richelson 1990; Sulser 1983*b*; Willner 1985; Crews et al. 1983; Marek and Seiden 1988*a,* 1988*b*). These compounds alter locomotor activity, food and water intake, and pain perception. In addition, they increase time spent in the forced swim test, increase the number of reinforcers obtained on a differential reinforcement of low rate 72-second (DRL 72-s) schedule, and have been reported to affect sleep. The above effects are consistent with the idea that 5-HT plays a role in the maintenance of physiological and behavioral function (Curzon 1990; Humphrey et al. 1990; Jimerson et al. 1990; Morin et al. 1990; Richardson 1990; Wauquier and Dugovic 1990).

Data from experiments with neurotoxins also suggest important behavioral functions for DA and 5-HT. Lesions to DA systems induced by 6-hydroxydopamine (6-OHDA) have profound behavioral effects shortly after the toxin is administered. These include severe decreases in food and water intake and locomotion. In addition, DA lesions alter the behavioral effects of drugs that act via DA mechanisms (Kostrzewa and Jacobowitz 1974). Depletion of 5-HT by neurotoxins or electrolytic lesions have been shown to have effects on sleep, reaction to painful stimuli, sexual behavior, and aggression, although there has been some difficulty in interpreting some of these findings (Seiden and Dykstra 1977). It is reasonable to suspect, therefore, that chronic depletion of DA or 5-HT would lead to long-term changes in behavior.

Methamphetamine (MA) and certain related phenethylamines are drugs of abuse that are toxic to DA- and/or 5-HT-containing neurons in the brain (Hotchkiss et al. 1979; Koda and Gibb 1973; Seiden et al. 1976; Seiden and Kleven 1989). When administered repeatedly to animals, these compounds induce long-lasting monoamine depletions on the order of 50 percent. It seems likely that these drugs are neurotoxic in humans. Therefore, it is important to determine whether monoamine depletions of the magnitude produced by these drugs have significant behavioral consequences. Although these compounds have been found to have little or no obvious long-lasting effect on behavior, behavioral testing has not been extensive. Therefore, the present series of experiments was designed to examine the effects of small-to-moderate depletions of DA and 5-HT induced by neurotoxins or neurotoxic amphetamines on a wide range of behaviors that were predicted to be sensitive to small changes in monoamine concentrations. Those behaviors included locomotion, feeding, drinking, avoidance, escape, and open-field testing as well as several different operant tasks that are known to be sensitive to antidepressant, anxiolytic, and antipsychotic drugs.

METHODS

Male Sprague-Dawley rats (3 to 4 months old) were housed individually in a temperature- and humidity-controlled facility on a 12-hour light-dark cycle (lights on at 7 a.m.). Rat chow and water were available ad lib, except in experiments with schedule-controlled behavior (fixed conservative number [FCN], variable interval [VI], and DRL) and eight-arm radial maze when the rats were maintained at 80 to 85 percent of their predeprivation body weights.

Neurotoxins

Rats were pretreated with pargyline (20 or 30 mg/kg, intraperitonally [IP] 60 to 75 minutes prior to the neurotoxin injections) and anesthetized with sodium pentobarbital (30 mg/kg, IP, 40 to 55 minutes prior) supplemented with methoxyflurane. Desipramine (20 mg/kg, IP) was administered 30 to 45 minutes prior to neurotoxins. 5,7-Dihydroxtryptamine creatinine sulfate (5,7-DHT) (25, 50, 75, or 100 µg/ventricle, as the base) (Regis Chemical Co., Morton Grove, IL) or 6-OHDA (25, 37.5 and 50 µg/ventricle, as the base) (Sigma Chemical Co., St. Louis, MO) was injected bilaterally intracerebroventricularly (ICV). Ampicillin (50 mg/kg, intramuscularly 25 to 40 minutes prior) was given to prevent infection. Postoperatively, the 6-OHDA lesion rats were given access to preferred foods (vanilla custard with whole milk and vanilla wafers) and gradually returned to standard chow.

Neurotoxic Drugs

(+)-MA HCI (12.5, 25, and 50 mg/kg/injection) or (±)-3,4-methylenedioxymethamphetamine (MDMA) HCI (MDMA) (10, 20, and 40 mg/kg/injection) was injected subcutaneously (SC), b.i.d. (morning and evening, 12 hours apart), for 4 consecutive days. Drugs were dissolved in 0.9 percent saline, and doses are expressed as the salts. Parachloroamphetamine (PCA) HCI was purchased from Sigma.

Behavioral Testing

A variety of behavioral tests were conducted. Methods and results from each test are described below. In most cases, testing began 2 weeks after treatment and continued for at least 2 weeks. The major exception was food and water intake for which one group was tested approximately 18 months after treatment.

Analysis of Monoamines

After behavioral testing, rats were sacrificed by decapitation and their brains were rapidly dissected over ice; the striatum, hippocampus, and frontal cortex were assayed for 5-HT and DA (Heffner et al. 1980). Concentrations of DA and

5-HT were analyzed using high-performance liquid chromatography with electronic detection, by the method of Kotake (Kotake et al. 1985).

RESULTS

Neurochemical effects of neurotoxins and neurotoxic drugs were dose related. Generally, only the maximum depletion that was observed is reported. Behavioral changes are summarized in table 1.

Locomotor Activity

Locomotor activity (Erinoff et al. 1979) was measured in a stabilimeter apparatus for a period of 24 hours (12 hours light, 12 hours dark) once per week in rats treated with 6-OHDA or 5,7-DHT and in appropriate control rats.

TABLE 1. *Summary of behavioral effects of neurotoxins and neurotoxic drugs*

	Treatment			
Behavioral Paradigm[*]	5,7-DHT	6-OHDA	MDMA	MA
Locomotor activity	0	0	—	—
Food intake				
Total (g)	0	ı	0	0
Number of meals	ı	0	0	0
Meal duration	0	j	0	0
Meal size	ϕ	0	0	0
Intermeal interval	0	ϕ	0	0
Water intake				
Total (mL)	0	0	0	0
Number of bouts	0	0	0	0
Bout duration	ϕ	0	0	0
Bout size	0	ı	0	0
Interbout interval	0	0	0	0
Schedule-controlled behavior				
FCN	0	0	—	—
VI 90-s	0	ϕ	0	0
DRL 72-s	ϕ	ϕ	0	0
Open-field behavior	0(ϕ)[†]	0(ϕ)[‡]	0	0
One-way avoidance				
Acquisition	0	0(ϕ)[‡]	0	0
Retention	0	—	—	—

TABLE 1. *(continued)*

Behavioral Paradigm*	Treatment 5,7-DHT	6-OHDA	MDMA	MA
Two-way avoidance acquisition	0[§]	0(ϕ)[‡]	0	0
Swim test	0(ϕ)[‖]	0	0	0
8-Arm radial maze				
Acquisition	0	—	0	—
Extinction	—	—	0	—
Morphine analgesia	i[**]	—	0[††]	0
Home cage intrusion	i	0	—	—

KEY: i = increased; 0 = no effect; ϕ = decreased/impaired; — = not tested.

NOTES:

* See Methods section above for abbreviations, doses, routes, and schedules of drug administration.

† A decrease in center squares entered was seen only in one experiment (Lorens et al. 1990) and only after the 50 mg, ICV total dose. This finding was not replicated nor observed at higher doses (100 to 200 mg, ICV).

‡ Alterations produced by 6-OHDA were not consistent across groups (see text).

§ PCA (5 mg/kg x 2d) also was ineffective.

‖ Impaired swimming ability was reported by Lorens and colleagues (1990) but was not replicated.

** Morphine (5.0 mg/kg, SC) analgesia was potentiated by 200 mg (ICV total dose) but not 50 mg 5,7-DHT. This effect was observed 8 but not 2 weeks postinjection.

†† This finding is in contrast to the report by Nencini and colleagues 1988.

No changes were observed in the face of an approximately 50-percent depletion of DA or 50 percent of 5-HT. Rats showed a low level of activity during the light and a slightly higher level during the dark.

Food and Water Intake

Food (45 mg BioServ dustless pellets) and water intake were measured using photo-beam detection units (Coulbourn Instruments, Lehigh, PA) and monitored by a computer in an adjacent room. Food intake was analyzed in terms of total grams, number of meals, meal duration, meal size, and intermeal interval. Water intake was analyzed in terms of total mL, number of bouts, bout duration, bout size, and interbout interval. The room was on a 12-hour light-dark cycle

and the last 5 days of stable intake were used for data analysis. After treatment, one group of rats (short term) was returned to the experimental chambers after a 2-week recovery period, for at least 2 weeks. A second group (long term) was singly housed and maintained at stable body weight (approximately 350 g) for 18 months, after which it was returned to the experimental chambers for at least 2 weeks, For both groups, the last 5 days of stable intake were compared based on drug dose received.

Short-Term Studies. In rats given 6-OHDA, DA was depleted to 44 percent of control in the caudate, 60 percent of control in the frontal cortex, 73 percent of control in the hypothalamus, and 63 percent of control in the hippocampus; DA was unaffected in the spinal cord. Total grams, meal duration, and bout size increased. Intermeal interval decreased. In rats given 5,7-DHT, 5-HT was reduced to 30 percent of control in the caudate, 14 percent of control in the hypothalamus, 7 percent of control in the hippocampus, and 16 percent of control in the spinal cord. 5-HT was not affected in the frontal cortex. Number of meals increased, whereas meal size and bout duration decreased.

The MDMA regimen depleted 5-HT to 61 percent of control in the caudate, 37 percent of control in the frontal cortex, and 49 percent of control in the hippocampus. 5-HT in other regions was unaffected. There were no effects on food intake. In rats given MA, DA was depleted to 70 percent of control in the caudate; DA in other regions was unaffected. 5-HT was depleted to 43 percent of control in the caudate, and 29 percent of control in the frontal cortex; it was unaffected in other regions. There were no changes in food intake.

Long-Term Studies. In rats given MDMA and tested approximately 18 months after the last injection, DA in the spinal cord was depleted to 55 percent of control, whereas DA in other regions was unaffected. 5-HT was not affected in any region. There were no changes in food intake. In rats given MA and tested approximately 18 months after the last injection, 5-HT was depleted to 16 percent of control in the frontal cortex and 26 percent of control in the hippocampus, increased to 131 percent in the hypothalamus, and was unaffected in other regions, DA was increased to 221 percent of control in the spinal cord and unaffected in other regions. There were no changes in food intake.

Schedule-Controlled Behavior

Three schedules of reinforcement were used to study the effects of neurotoxins on schedule-controlled behavior: an FCN, a VI 90-second (VI 90-s), and a DRL 72-s. After stable behavior was established under each schedule, neurotoxins were administered. Retesting began approximately 2 weeks after administration of neurotoxins.

Fixed Consecutive Number

The FCN was conducted in standard two-lever operant chambers (Gerbrands Corp., Arlington, MA). Five or more consecutive responses on the left lever followed by one response on the right lever were required for delivery of a food pellet (45 mg, Noyes). A response on the right lever before five consecutive responses on the left lever reset the response requirement on the left lever. This schedule contained a signalled (SD) and an unsignalled (SO) component; each component consisted of 10 trials. The components (SD-SO) alternated until a total of 10 components (5 each) were completed. During the SD, the left lever light was illuminated until five consecutive responses were made; then the left lever light was extinguished, and the right lever light was illuminated. During the SO component, only the houselight was illuminated. The probability of reinforcement during the SD component was adjusted so that the number of reinforcers for both components was approximately equal. Responding was analyzed as percent correct for each component.

In rats that received 6-OHDA, DA was depleted to 14 percent of control in caudate, increased to 172 percent of control in the spinal cord, and was unaffected in other regions. There was no effect on FCN performance. In rats that received 5,7-DHT, 5-HT was depleted to 20 percent of control in caudate, 16 percent of control in frontal cortex, 34 percent of control in hypothalamus, 12 percent of control in hippocampus, and 15 percent of control in the spinal cord. DA was reduced to 76 percent of control in caudate, 51 percent of control in hypothalamus, and 14 percent of control in spinal cord; it was unaffected in other regions. NE was depleted to 4 percent of control in the hippocampus but was unaffected in other regions. FCN performance was unaffected.

VI 90-s responding (Levine et al. 1980*a*, 1980*b)* was unchanged following approximately 50-percent depletions of either DA or 5-HT. The rates of both responding and reinforcement were the same between the treated and the sham-treated rats.

Responding under the DRL 72-s was also unchanged (response rate and reinforcement rate did not differ) as a result of approximately 50-percent depletions of either DA or 5-HT.

Behavioral Test Battery

An additional battery of behavioral tests, designed to assess various aspects of behavioral function, was used to examine the effects of neurotoxins and neurotoxic drugs. The test battery included open-field behavior, one-way avoidance, discriminated two-way avoidance, and the forced swim test (Lorens et al. 1990). The effects of both neurotoxins and neurotoxic drugs were examined using this test battery. The effects of 5,7-DHT and MDMA on

eight-arm radial maze performance and morphine-induced analgesia also were determined. In addition, the effects of 6-OHDA on a home-cage-intrusion test were examined. Groups of rats were treated with neurotoxins and neurotoxic drugs and evaluated in multiple tests.

In rats that received 5,7-DHT, 5-HT was depleted to about 20 to 40 percent of control. MDMA also produced dose-dependent and selective reductions in regional CNS 5-HT concentrations that were virtually equivalent (18 to 34 percent reductions) in all CNS regions assayed (catecholamine concentrations were not altered). The lowest dose (50 µg) of 5,7-DHT significantly decreased open-field behavior and impaired swimming ability. MDMA did not alter open-field behavior or swimming ability. There was no effect of 5,7-DHT or MDMA on one-way (spatial, unsignalled) or visually discriminated two-way (nonspatial, signaled) avoidance conditioning. Additionally, neither treatment affected acquisition of one- or two-way avoidance or performance in the eight-arm radial maze.

Although dose-dependent effects of 6-OHDA were obtained, the changes in regional DA concentrations were highly variable. Thus, the 6-OHDA-treated rats were grouped according to their caudate-putamen DA levels: 6-OHDA-1, 83 percent of control: 6-OHDA-2, 66 percent of control; and 6-OHDA-3, 34 percent of control. In the frontal cortex, DA reductions reached 56 percent, but the variability precluded statistical significance. Decreases in hypothalamic DA were more modest but statistically significant. No systematic alterations were detected in hippocampal or spinal cord monoamines and their metabolites. Some significant behavioral changes were observed that varied as a function of lesion group and test. In the open field, the 6-OHDA-2 group exhibited a reduction in center squares entered. The 6-OHDA-3 group made more errors during one-way conditioned avoidance response (CAR) training, whereas the 6-OHDA-1 group returned to the shock compartment (errors of commission) more often during two-way CAR acquisition. There was no effect in the swim test, and there were no changes in aggressive or social behavior in the home-cage-intrusion test.

In rats treated with MA, DA levels were reduced only in the neostriatum (by 34 percent). Additionally, MA reduced 5-HT in the frontal cortex (by 61 percent), neostriatum (by 57 percent), hippocampus (by 81 percent), and spinal cord (by 29 percent) but not in the hypothalamus. NE concentrations were not altered in any CNS region. There was no effect on open-field exploration, the acquisition of a one-way or visually discriminated two-way CAR, swimming ability, or morphine analesia (5 mg/kg, SC).

DISCUSSION

An overview of the behavioral results obtained in the present series of studies shows that although neurotoxins had some long-lasting behavioral effects (e.g., food intake, VI and DRL performance), a wide variety of behaviors were unaffected by partial denervation of the DA or 5-HT systems by MDMA or MA. In addition, some of the changes that were observed with neurotoxins were generally unsystematic with regard to dose and, therefore, may have been the result of random variation. One might draw the conclusion from these results that DA and/or 5-HT do not play an important role in mediating these behaviors. However, these results should be interpreted with caution because the effects of drugs on 5-HT and DA systems strongly suggest that these systems play a role in behavior.

An account of the results presented here must, at this point, remain largely speculative. It may be that the functional reserve capacity of the CNS readily compensated for neurotransmitter changes. Within a particular neurotransmitter system, the amount of release that is compromised by drug-induced toxicity may be too small to be reflected in behavior. There is some evidence to support this view in that larger depletions of DA or 5-HT cause more persistent changes in behavior. VI 90-s responding has been found to increase with time as a result of 6-OHDA lesions that depleted both DA and NE by greater than 70 percent. Similarly, large depletions of DA cause response rate increases and reinforcement rate decreases in DRL 72-s behavior, and 5,7-DHT lesions that produce a large depletion of 5-HT increase the response rate and decrease the rate of reinforcement on the DRL 72-s schedule.

Alternatively, it may be that the behaviors studied, rather than relying on the function of any particular neurotransmitter system, rely on several synergistic transmitter systems. A partial reduction in the presynaptic terminal field of one neurotransmitter may be readily compensated for by one of the synergistic systems.

Other possibilities involve functional compensation in CNS neurotransmission. It may be that transmission in DA and 5-HT neurons was normal or at least adapted to maintain normal behavioral function. Although the concentrations of monoamines in the brain were decreased, this measure may not reflect the amount that is released. If synthesis and release were increased as a compensatory mechanism, then the same number of receptors would be stimulated and function would not change. Although it is difficult to understand how postsynaptic tone would be maintained if one assumes that the DA and 5-HT systems comprise tight chemical junctions, the assumption of volume transmission makes the results more reasonable. In volume transmission, the

released transmitter can diffuse many millimeters from the release site and thereby stimulate receptors not located within microns of the presynaptic terminal. Thus, one could readily see why function would not be compromised even in the face of significant decreases in the levels of DA and/or 5-HT. It is also possible that the number of receptors changed in such a way as to give a normal response in the face of only partial agonism. These events do occur in the face of denervation, but there have not been adequate studies to determine how these changes translate into effects on brain function and behavior.

Regardless of the precise mechanisms involved, one is left with the somewhat surprising conclusion that drug-induced CNS monoamine neurotoxicity had few, if any, long-lasting behavioral consequences in these studies. However, this should not be construed as meaning that CNS neurotoxicity is inconsequential. It is possible that the behavioral tasks used were not sensitive to functional changes. Moreover, behavioral consequences of neurotoxicity may be more apparent under different conditions. For example, depletions of DA in neonatal rats have been shown to cause hyperactivity that persists into adulthood (Erinoff et al. 1979). It may also be the case that the appearance of age-related deficits is hastened by drug-induced neurotoxicity. It remains for future research to examine these possibilities.

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DISCUSSION

Q: I was wondering if you saw any long-term changes in body weight?

A: Not when they are compared with their own rate of growth. In other words, you would get some initial losses, particularly with methamphetamine [meth], in the body weight, but then if you compared them with controls in a relatively free-feeding situation, they will gain weight at about the same rate as the controls. So it does lag a little bit.

Q: Second question: On one of your slides you said something about the recovery of the serotonin, and I was wondering, are those changes that you were talking about relatively permanent or were you looking at them in some point in the recovery phase?

A: Now, that is a good question. It depends upon which drug you are talking about. For the meth, we've looked for long times after we have treated these animals, and they are down in serotonin, and they are down in dopamine. But for fenfluramine, we do see a certain amount of recovery in certain regions in the brain as we do for MDMA; furthermore, it depends on what brain region you are talking about and at what aspect of the system you are talking about. Mainly, we look at levels, and the levels will tend to come back toward normal in certain brain regions and stay flat in other brain regions. Dr. Molliver will talk about this in more detail: there seems to be some recovery in levels. Nate Appel has seen some recovery in the uptake sites. It is a matter of controversy as to how to interpret some of these data, and maybe we can get some of that worked out here.

Q (Gibb): Lew, having spent my life looking at changes in neurochemistry, I find your results discouraging. On the other hand, the fact is that we do know that fluoxetine is effective in the treatment of depression. Also, as I understand it, it is very effective in the treatment of obesity. If my information is correct, the primary focus of that drug's effect is on the serotonergic system. So I would conclude that 5-HT is important, even though at this point none of the data that we have available from animal studies would suggest that. That would be the skeptic's view. It seems to me that the question that really needs to be addressed now (and I think you have said it) is whether the tests that we are using are sophisticated enough to pick up those kinds of changes. That is the important issue here. You would think that the feeding behavior would address the issue. Obviously, I am not a behaviorist, and I wondered if you care to comment on that?

A: The project took a period of 3 years, We honestly thought that the tests that we were using would be sensitive enough to pick up changes as they occurred.

Q: Have you looked at sex at all? Because as I understand it, I don't know. . .

A: Lorens looked at sexual behavior, but he had a very limited trial. And there were no changes.

Q (Heimer): Lew, I was wondering about the definition of the term "neurotoxicity" in relation to the techniques you have covered here. I suppose that you take for granted, as does everybody who uses the so-called "suppressive" silver methods, that when you see heavily argyrophilic particles or a strongly argyrophilic neuron, this neuron is degenerating and will eventually

die. When you do see black “measles” representing degenerating terminals, you take for granted that they will die and that the related neuronal cell bodies will also die. Although many people have been obsessed by silver methods, there are only a few who have been experimenting with more and more refined techniques. Maybe you saw the material at the Anatomy Meeting that Dr. de Olmos and his colleagues from Argentina produced. They kept modifying their silver technique to the point where, in quinolinic acid experiments, they used the same dose that people have used to make “restricted” lesions in the striatum. De Olmos and his colleagues detected degenerating neurons not only in striatum but in cerebral cortex, thalamus, and various other places even 15 minutes after the injection. I was just wondering if the silver methods have become so sensitive that they might even pick up changes that are reversible. I am sure that there are many who know this area better, so they might even have thought about these things, but as I remember from the time I used silver techniques, there are few people studying the ultrastructure of these argyrophiiic particles and neurons. I wonder what you think about this problem?

A: George Ricaurte spent a lot of time working at the level of the electron microscope and did most of the silver staining procedures along with Ray Guillery. I thought that the changes using the electron microscope were not reversible. I must say that, since that time, Phil Groves has obtained evidence showing very nicely that you can see a silver-positive neuron in a cell that has been stained immunologically for tyrosine hydroxylase, indicating that it is the same cell that takes up silver. Now, if you combine that with the idea that the uptake site is down and that the levels are down, I think the conclusion becomes inescapable. But I would agree with you that any single benchmark in and of itsetf may not be a hallmark for neurotoxicity. We have converging evidence from chemistry, from function (if you take uptake as a function of the ceil), and the argyrophilic nature of the fiber that degeneration can occur. But taken singly, I think that any one of these things could lead us astray.

Q (Molliver): About 10 years ago, S. Ögren and colleagues published a series of reports on animals treated with PCA [parachloroamphetamine] that have a selective serotonin deficiency. They found profound behavioral changes: a tenfold decrease in conditioned avoidance learning. Would you comment on these data?

A: We have never looked at it using PCA. We have looked at conditioned avoidance acquisition using other 5-HT toxins, but we have not been able to replicate these data; but we have never tried it with PCA.

Q (Molliver): That was a profound effect. Have others been able to replicate these data?

A: I don’t think so. I think they have tried.

Q (Gibb): Lew, one other thing that I think needs to be taken into consideration here is (I am sure George will tell us about this) that the rat is relatively less sensitive to these compounds than primates. And I wonder, therefore, if there is any possibility that there is impairment of behavior in primates subsequent, down the line. What I think your data show is that there is a tremendous amount of plasticity in the system. And we know that if you use 6-hydroxydopamine, you have to deplete more than 80 percent or so of the dopamine to show any parkinsonian symptoms. I wonder if the same thing applies here, and if so, is it worthwhile conducting tests. I guess you couldn't do it in rats, maybe, but to see whether with age there is a deficit that develops. I think the concern that all of us have with regard to the abuse of these compounds is what is going to happen to these individuals in their sixties or earlier, possibly, as they abuse these amphetamine compounds; will they be early parkinsonian patients? Is there anything that we can do as far as tests are concerned?

COMMENT (Schuster): We did look extensively at the behavior of the primate after serotonin and dopamine depletions induced by high levels of methamphetamine. And I must say that we were as completely disappointed in those investigations as Lew had been with these more recent rat studies. Our studies used very sophisticated behavioral methods such as force-lever techniques in which you are able to measure hand tremor, and despite the fact that the monkeys had 60 to 70 percent depletion of dopamine, we did not see any changes in their performance.

COMMENT (Ricaurte): It is a nice idea. However, I actually favor the other possibility you mentioned, and that is that there are functional deficits but that you need the right task and a sufficient degree of damage within a given neurotransmitter system to detect it. So, for example, in the case of the force-lever paradigm, I would maintain that the animals didn't have as much dopamine depleted from the basal ganglia that one would have liked; I also would maintain that perhaps the task, although it is a motor task, may not be the appropriate task to get at extrapyramidal motor dysfunction. Before going on to other systems, I would favor the notion of stressing a given system and selecting the appropriate task to see whether it is possible to detect subclinical deficits.

A (Seiden): These monkeys were impaired when they were challenged with physostigmine, as I recall.

Q (Molliver): I have a question about Dr. Gibb's comment regarding fluoxetine. You must continue to give fluoxetine to see its effects on the depression, so this is a pharmacological action as opposed to a permanent alteration induced by the given compound. If you give MDMA every day, you will see behavioral

changes. However, if you give a course of it, wait for 3 weeks, and then look at the system, you don't see any permanent changes in behavior.

A (Seiden): That takes you back to the dilemma again. If you are modifying the presynaptic level pharmacologically and you get a change, and then you deprive the system of the presynaptic transmitter and you don't get a change, I have trouble interpreting those data.

Q (Landfield): I think this issue you raised on effects in animals is really very Important, and it is going to be a recurring issue because we see the same thing in our THC [tetrahydrocannabinol] studies-the same issues. There are two factors that make me think that one shouldn't be too discouraged about the discrepancies. We have to remember that human behavior has a lot of redundancy built in, a lot of subtlety. Even in the case of Aizheimer's disease, there has to be massive neuronal degeneration before a patient is institutionalized. Human behavior is much more complex; at least, we like to think so. We can see subtle differences in human behavior that we might not see in the rat behavior. A few cocktails can change people's speech: we can notice changes. Whereas to observe an alcohol effect on behavior in a rat, you might need to induce a level of alcohol toxicity that would have a human completely knocked out. We have to remember that with human behavior, we can detect very subtle changes. The ratio between toxicity and observable behavioral effect may be very different in different species. Now, on the other side of the coin, some conditions allow us to detect a larger effect in a rat. In rats, one can damage the nervous system and see much more degeneration from certain toxic drugs because of experimental design: for example, dosage can be controlled to obtain a much higher rate of delivery. As you said earlier, if you don't see that effect in humans, it can be disappointing or seem less Interesting. However, that may not be so, because toxicity in animals can at least provide clues to which brain systems are being changed by the drug or what is happening to membranes. These toxic effects in animals can provide clues to mechanisms, even though they are not seen in humans. You might have to treat for 20 years in humans to see the same effect. We also have to remember that the lifespan of the rat is 2 years, so everything is telescoped. What takes 2 months in a rat may take 10 years in human beings. I don't think we should be disappointed about discrepancies-just recognize the large differences in the experiments with different species. The discrepancies are providing important clues, and we shouldn't expect to see the same things in rats and humans exactly. I think the points that were made here are very important. You need different types of tests, more subtle tests, to see subtle changes in rat behavior.

A (Seiden): I couldn't agree more. I think that is exactly what I wanted to do here. I think that I've stimulated our discussion. Tom?

Q (Sobotka): Could I suggest looking at more local areas of the brain for depletion? I was working in the area of norepinephrine and noticing that Dr. Molliver and his colleagues have shown us that there is quite a bit of differentiation in where norepinephrine was located in the brain. I tried to locally deplete norepinephrine in rabbits. At least with conditioned avoidance tests, many people have looked for behavioral effects of depletion of norepinephrine, and there really isn't a lot of consensus in that area. I thought that since you tried a global depletion, there may be some kind of global compensation. Norepinephrine and serotonin are interacting with many other things locally in the brain, so what if we depleted norepinephrine locally and found significant behavioral changes in our task?

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Strategies for Detecting Subclinical Monoamine Depletions in Humans

Una D. McCann and George A. Ricaurte

INTRODUCTION

Certain lesions of the central nervous system (CNS) have obvious manifestations that are readily detected by an experienced clinician. For example, a right-handed individual who develops sudden right-sided weakness and language problems is likely to have pathology involving the left cerebral hemisphere. A careful neurological examination might yield more specific information regarding the anatomical distribution of the lesion, which often can be confirmed radiologically using imaging techniques such as computerized tomography or magnetic resonance imaging (MRI). Unfortunately, other lesions of the CNS may remain undetected for long periods, both because they do not have obvious clinical manifestations under ordinary conditions and because behavioral functions sewed by the involved brain areas may be subtle and difficult to test and measure.

Parkinson's disease, a neurodegenerative disease involving brain dopamine neurons, provides an example of a naturally occurring CNS lesion that can exist in a preclinical or asymptomatic form and that can go undetected for years (Koller 1992). Patients with late-stage Parkinson's disease are readily diagnosed by a well-defined symptom complex that includes slow movement, rigidity, resting tremor, and postural instability. Often, there is also a loss of facial expression and some degree of cognitive impairment. However, none of these impairments becomes apparent until 60 to 70 percent of dopamine neurons have degenerated and brain dopamine concentrations are depleted by 80 to 90 percent (Hornykiewicz 1975; Forno and Alford 1987; Koller 1992). The absence of symptoms in the face of such large dopamine depletions underscores the problem of preclinical diagnosis. This problem is magnified severalfold when brain structures with more subtle functions (e.g., serotonin or mesocortical dopamine systems) are involved.

Several recreationally used amphetamine analogs, including methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyethylamphetamine (MDEA), have the potential to

damage CNS dopamine and serotonin neurotoxins in a variety of animal species (Seiden and Ricaurte 1987). Whereas the toxic effects of MA involve dopamine and serotonergic neurons, the toxic effects of MDMA appear to be specific for serotonergic neurons (Stone et al. 1986; Battaglia et al. 1987; Ricaurte et al. 1988*a*). Moreover, it appears that nonhuman primates are at an increased risk for developing MDMA neurotoxicity (Ricaurte et al. 1988*b;* Ricaurte and McCann 1992), and there is increasing concern that humans who use amphetamines recreationally may also incur damage to brain dopamine and serotonin neurons. Because the recreational use of MDMA and related drugs may be on the rise (Noticeboard 1992), it is important to determine their neurotoxic potential in humans and develop reliable methods for detecting preclinical monoamine neurotoxicity, if it exists. This chapter reviews the strategies available for detecting partial depletions of CNS dopamine or serotonin in humans. Strengths and weaknesses of each strategy are discussed, and potential tools for enhancing current methodologies are proposed.

CEREBROSPINAL FLUID ANALYSIS

Cerebrospinal fluid (CSF) bathes the brain and spinal cord. Because of the CSF's close proximity to brain structures and its isolation from other major organs, changes in the neurochemistry of CSF are thought to reflect changes specific to the CNS (Wood 1980). Furthermore, because the total volume of CSF (100 to 150 mL) undergoes complete replacement approximately six times per day (approximately 600 to 700 mL per day), CSF measures can be used to detect relatively short-term dynamic changes in the CNS milieu. Although plasma and urine measurements also have been utilized as indices of CNS monoamine neurotransmitter function, these are generally considered to be less specific because multiple organ systems (including the adrenal medulla) contribute considerably to their components (Kagedal and Goldstein 1988).

CSF measures not only are used routinely for the determination of neuropathological processes in clinical populations but also have been used as a strategy for detecting suspected neurotransmitter alterations in neuropsychiatric diseases. More specifically, CSF collected by lumbar puncture has been used to measure concentrations of relevant neurotransmitter metabolites (Moir et al. 1970). With regard to amphetamine neurotoxicity, measurements of lumbar CSF homovanillic acid can be used as an indirect measure of brain dopamine activity (Kagedal and Goldstein 1988), whereas 5-hydroxyindoleacetic acid (5-HIAA) can be used to detect changes in brain serotonin (Ricaurte et al. 1988*a;* Banki and Molnar 1981). Decreases in either

of these metabolites in individuals who had been exposed to amphetamines provides support for the occurrence of amphetamine neurotoxicity in humans.

The advantages of using CSF measures to detect subclinical neurotoxicity include easy accessibility of CSF, reproducibility of the assay (Menachem et al. 1989) and that it is a dynamic measure. Drawbacks of this strategy include that CSF composition provides an indirect reflection of brain neurochemical activity, that it is nonspecific (numerous factors are known to influence the composition of CSF) (Moir et al. 1970; Post and Goodwin 1977; Wood 1980), that it is not particularly sensitive (i.e., concentration changes in the cervical spine underestimate changes in the forebrain) (Sourkes 1973; Sjostrom et al. 1975; Ricaurte et al. 1988*a),* and that the lumbar puncture procedure can be unpleasant and can have complications (e.g., “lumbar puncture headache”).

NEUROENDOCRINE STUDIES

A second strategy for detecting subclinical damage to dopamine and serotonin systems takes advantage of the important roles of these systems in the hypothalamic-pituitary-adrenal (HPA) axis. Both dopamine and serotonin are involved in the normal regulation of prolactin and growth hormone secretion (for reviews, see Kato et al. 1985; Arimura and Culler 1985) and damage to either monoaminergic system can sometimes be detected using neuroendocrine measures. For example, dopamine is known to tonically inhibit prolactin release at the level of the pituitary (Weiner and Bethea 1981). When dopamine levels are severely depleted, or postsynaptic dopamine stimulation is decreased, abnormally high baseline prolactin levels result. In instances where depletion of dopamine is not extensive, it may be necessary to pharmacologically challenge the dopamine system before endocrine disturbances are noted. For example, in a normal individual, an oral dose of L-dihydroxyphenylalanine (L-dopa), the precursor to dopamine, leads to a rise in growth hormone levels. In an individual with damaged dopamine neurons, there might be a blunted growth hormone response to L-dopa because fewer dopamine neurons would lead to reduced conversion of L-dopa to dopamine. By contrast, the same individual given a postsynaptic dopamine receptor agonist (e.g., bromocriptine) might be expected to have an abnormally robust rise in growth hormone, because of postsynaptic dopamine receptor supersensitivity.

In the case of serotonin, similar neuroendocrine strategies have been utilized to measure neurotransmitter dysfunction. Because serotonin plays a role in regulating normal secretion of prolactin, growth hormone, and cortisol, damage to serotonin pathways in the HPA axis can be detected through disturbances in the homeostatic mechanisms of these hormones (Heninger et al. 1984). For

example, both direct and indirect serotonin agonists (e.g., m-chlorophenylpiperazine and L-tryptophan) cause acute elevations in serum prolactin concentrations (Meites and Sontag 1981; Charig et al. 1986), possibly via a prolactin-releasing agent at the level of the hypothalamus (Garthwaite and Hagen 1979). In an individual with severe serotonin damage in the hypothalamus, the rise in prolactin following administration of L-tryptophan might be expected to be diminished (assuming that postsynaptic supersensitivity is not sufficient to mask presynaptic dysfunction). If the damage is partial, or if a postsynaptic 5-hydroxytryptamine agonist is administered, a supraphysiologic response might be anticipated. Because of the possibility of compensatory mechanisms over time (e.g., postsynaptic receptor supersensitivity or decreased inhibitory feedback), timing of the neuroendocrine challenge relative to the time of the suspected neurotoxic insult may be crucial for proper interpretation of the neuroendocrine challenge.

The previous examples of neuroendocrine methods for detecting preclinical monoaminergic damage are not exhaustive and are intended to illustrate the general neuroendocrine strategy. The advantages to neuroendocrine studies include their sensitivity, low cost, relatively noninvasive nature, and the ease with which procedures can be performed. However, like CSF measures, neuroendocrine measures are indirect and nonspecific. Furthermore, because of the possibility of compensatory mechanisms, results of the neuroendocrine challenge may not be consistent over time. Therefore, although potentially lending support for the diagnosis of neurotoxicity, neuroendocrine disturbances are not definitive.

NEUROIMAGING TECHNIQUES

Recent advances in imaging technology and the development of neurotransmitter-specific ligands hold promise for the detection of subclinical neurotoxic injury. As further advances are made, imaging techniques that can be used to this end will include single photon emission computerized tomography, positron emission tomography (PET), and possibly MRI (at least in cases where the extent of neuronal volume loss is sufficient). However, to date only PET has been used successfully in humans for the detection of neurotoxic damage, and only in the dopamine system (Calne et al. 1985). The reasons for this have been largely caused by the limited number of suitable ligands available rather than because of limitations of imaging techniques. However, in the case of serotonin, which is present in the brain at much lower concentrations than dopamine, sensitivity of imaging techniques also may prove to be problematic in detecting subtle neurotoxic lesions.

Because it is known that neurotoxic amphetamines damage the presynaptic element, the ideal ligand for detecting amphetamine neurotoxicity would be a presynaptic marker. Postsynaptic receptor markers, although possibly demonstrating receptor down- or up-regulation, would not provide compelling evidence of neurotoxicity. Of the various possible presynaptic markers, ligands for the uptake site may prove to be superior for the detection of neurotoxic injury because they label a "structural" macromolecular element of the nerve terminal (as opposed to neurotransmitter precursors such as 6-fluorodopa that can be altered by metabolic events). In the case of the dopamine system, several such ligands exist and include GBR-12909, nomifensine, and WIN-35428. Neurotoxic damage in humans caused by the potent neurotoxin 1 -methyl-4-phenyl-1,2,3,8-tetrahyropyridine has been detected using 6-fluorodopa, and this ligand also should be useful in detecting dopaminergic damage caused by amphetamines such as methamphetamine, which damages dopamine nerve terminals.

Development of presynaptic markers for serotonin neurons has been more difficult, although there are several promising candidates in the development phase. In particular, a variety of ligands that label the serotonin transporter complex (e.g., paroxetine derivatives) may prove to be ideal for studies in individuals exposed to potential serotonin neurotoxins such as MDMA or MDEA.

Advantages to PET and other neuroimaging techniques are that they provide evidence of neuronal alteration, they can be anatomically and pharmacologically specific, and they are relatively noninvasive (intravenous catheters are required). Disadvantages to neuroimaging studies are their potential lack of sensitivity, their expense, the large number of personnel required for completion of the study, and the inability to perform studies without special equipment and personnel.

PHARMACOLOGICAL CHALLENGE

A specific pharmacological challenge to a compromised neurotransmitter system has not been widely used in the detection of subclinical monoaminergic neurotoxicity but has potential utility. This method would take into account that the neurotransmitter system in question, although not eradicated, has limited reserves. In essence, under normal conditions, a compromised neurotransmitter system might be able to function, but if it is stressed, abnormalities might become apparent. An analogy can be drawn with the use of the cardiac stress test in instances where coronary artery insufficiency is suspected but unconfirmed. In an individual with "silent" coronary artery disease, cardiac abnormalities are inapparent under ordinary physical

conditions. Abnormalities such as irregular heart rhythms or angina pectoris may become manifest when the Individual is subjected to physical stress such as the treadmill test (Astrand 1978; Kilpatrick 1986).

A pharmacologic neuronal stress test might be useful in the evaluation of patients with suspected subclinical neurotoxicity. For example, in an individual with a history of significant methamphetamine use, diminished dopaminergic neurotransmission might be expected but, under ordinary conditions, might be clinically inapparent because it was not sufficiently severe. If that individual were given a drug that specifically interfered with dopaminergic neurotransmission, it might be anticipated that the subject would demonstrate exquisite sensitivity to its effects. Drugs that could be used to challenge the dopaminergic system include α-methyl-para-tyrosine, a synthesis inhibitor, or haloperidol, a postsynaptic dopamine receptor blocker. Endpoints that could be used to measure drug effects include parkinsonian symptoms and elevations in serum prolactin levels, as mentioned above.

Specific challenges of the serotonin system might be accomplished using the serotonin synthesis inhibitor parachlorophenylalanine. Unlike challenges to the dopamine system, challenges to the serotonin system have no obvious and easily measured functional endpoint. However, because serotonin has been implicated in the regulation of sleep, mood, anxiety, impulsivity, and pain sensitivity, disturbances in these behavioral spheres might be expected.

SUMMARY

Given the reported increase in the recreational use of controlled substance analogs such as MDMA and related drugs, it is important to determine whether these drugs produce neurotoxic effects in humans. Several strategies available for detecting preclinical neurotoxicity to dopamine and serotonin neurons have been discussed, and their strengths and limitations have been listed. In addition, some promising strategies that are still in the development stage (e.g., PET) have been mentioned. None of the available methods for detecting neurotoxicity is conclusive: therefore, converging lines of evidence will be essential to provide convincing indication of subclinical neurotoxicity in humans. Such studies will help define the public health risk of recreationally used drugs. Furthermore, documentation and determination of drug-induced neurotoxic changes may shed light on the pathophysiology of idiopathic neurodegenerative diseases involving monoaminergic neurons in humans and could be useful in the development of new treatment strategies. Finally, detailed neuropsychiatric evaluation of individuals with confirmed subclinical serotonergic neurotoxicity may enhance knowledge regarding the functional role of brain serotonin neurons in health and disease.

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DISCUSSION

Q: George, do you think that we will ever be able to get a clear answer by comparing people who use MDMA with control groups? Are we going to need to use people as their own controls?

A: If we compare results of an MDMA cohort with those of a control group, the comparison is compromised for a number of reasons, including the fact that invariably people have used other drugs as well. Also, there may be differences in educational background as well as a host of other differences that make intergroup comparisons difficult. One strategy that might be used to get around such problems would be an epidemiologic one, where numbers are such that you can begin to factor out some of the variables; but still the evidence is going to be indirect. Between-group comparisons are flawed, but I am afraid for now it is really the only viable option given the fact that to try to do before-and-after comparisons using subjects as their own controls is difficult unless (1) you have the authorization to do such a study and (2) you are willing to do such a study because (as you know) there are risks associated with testing the effects of MDMA on humans.

Q: How far do you think you have to go without doing that?

A: If you can develop a consistent line of argument with numerous lines of evidence where various results all point to the same conclusion, I think you can go reasonably far. I doubt that we will ever reach the point where we can definitely state that there is such a percent depletion of serotonin in a given individual's brain. However, using the strategies I have discussed in combination, looking for converging lines of evidence, I think you can probably develop a reasonable case for the presence or absence of neurotoxicity in a human cohort. In that regard, the human situation is not terribly different from the preclinical situation, where converging lines of evidence also are necessary to reach the conclusion that you are in fact dealing with neurotoxicity. None of the measures is going to make a compelling case by itself. But if different measures are considered together, a picture begins to emerge.

Q (Miller): Has anyone tried to look at the same issues with the fenfluramine populations in Europe?

A: Not that I know of. There is one disturbing result in the literature. Fenfluramine is often used instead of L-tryptophan as a challenge agent to study serotonin systems in humans. The disturbing observation is that when Coccaro and colleagues' used fenfluramine as the challenge drug, they found a shift in the baseline of their "control group," so that when a control group was rechallenged with fenfluramine 1 week later, the prolactin response appeared blunted, suggesting a change in serotonin system as a function of fenfluramine exposure. Aside from that one study, fenfluramine-exposed individuals have

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[1] Coccaro, E.F.; Siever, L.J.; Klahr, H., et al. Diminished prolactin responses to repeated fenfluramine challenge in man. *Psychiatry Res* 22(3):257-259, 1987.

not been studied either with CSF or neuroendocrine challenge strategies, to my knowledge.

COMMENT (Seiden): There was one study done in Europe that was published in Lancet, I think last year-a fairly large study of people who were getting fenfluramine for a whole year. They were testing various neurological and psychological outcomes in these patients who had taken fenfluramine for weight reduction. There was nothing marked in that study. But the only way a factor would come up in that study was if the patient reported to the physician that he was experiencing x, y, or z effect. Patients weren't given questionnaires. So it is a little biased toward nonreporting.

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Sensory-Evoked Potentials: Measures of Neurotoxicity

William K. Boyes

NEED FOR TESTS OF SENSORY FUNCTION

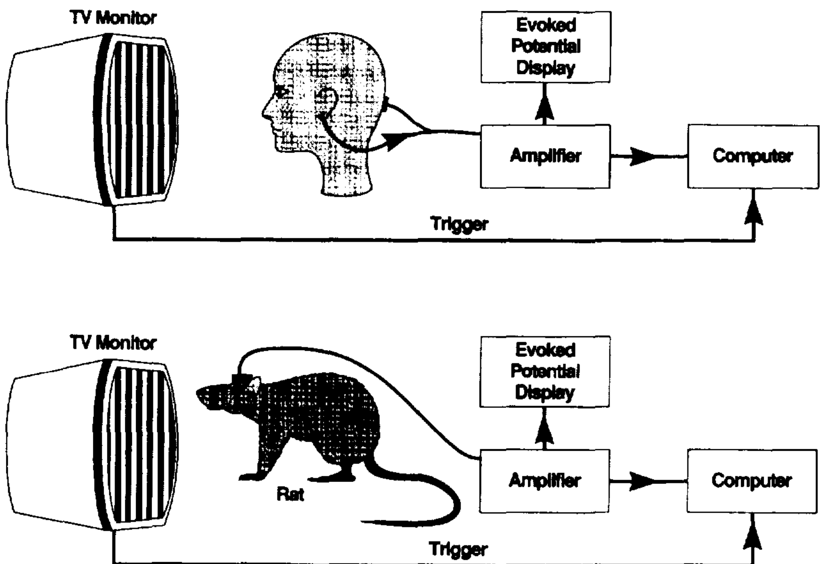
Only a portion of approximately 65,000 chemicals used in commerce have an adequate toxicity database (Office of Technology Assessment 1990). Although no hard figures are available, it has been estimated that approximately 25 percent of the compounds tested produce significant neurotoxicity (Office of Technology Assessment 1990). Of those compounds reported to have neurotoxic effects, one analysis found that approximately 44 percent produced sensory system effects, with visual effects being most common (Crofton and Sheets 1989). Combining these values would yield an estimate, admittedly crude, that approximately 11 percent of all compounds are potential sensory toxicants. In comparing the types of visual system effects reported in humans with those observed in laboratory animals, the human reports are more likely to involve loss of visual perception, such as blurred or double vision, whereas the animal reports are more likely to be easily observable lesions such as cataracts (Boyes 1992). Rarely are deficits of sensory function reported in laboratory animal toxicology studies. The reasons for this are fairly obvious, because animals do not readily reveal what they see, hear, or feel. Furthermore, animals have not been "asked" these questions with technology capable of providing some of the answers. Given the likelihood that many chemicals are toxic to sensory systems, it is necessary to develop and implement procedures that will enable the detection and characterization of sensory toxicity in routine animal toxicology studies. At the same time, it would be advantageous to obtain information about mechanisms of action, perception, and the comparability of responses in laboratory animals and humans.

SENSORY-EVOKED POTENTIALS

The analysis of sensory-evoked potentials (SEPs) has much to offer in filling such needs. These responses are averaged electrophysiological events recorded from appropriately positioned electrodes following repeated presentation of sensory stimuli. Typical recording situations for measuring

visual-evoked potentials from human and rat subjects are depicted in figure 1. A sensory stimulus, in this case a changing visual pattern, is presented to the subject. Electrical activities recorded from electrodes positioned over the primary visual projection areas are differentially amplified with respect to a reference site, and several responses are averaged together by computer in synchrony with stimulus presentation. Thus, activity that is unrelated to stimulation averages out, whereas activity that occurs repeatedly in a time-locked fashion following stimulus presentation is enhanced. The resulting

Pattern-Evoked Potential Recording Procedure



Pattern Onset-Evoked Potentials

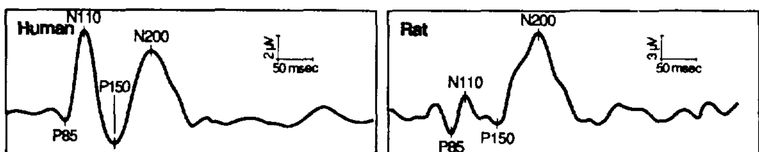


FIGURE 1. *Typical evoked potential recording arrangements for humans and rats, along wifh pattern onset-evokedpotentials recorded from eachspecies*

averaged evoked potential reflects summed neural responses involved in processing sensory input. A variety of sensory modalities can be studied, including vision, audition, and somatosensation, which yield visual-, auditory-, or somatosensory-evoked potentials, respectively. Variations in the stimulus parameters, and in the recording procedures, can be utilized to obtain information about the operational characteristics of the neural systems that process stimulus information. Evoked potentials can be used in conjunction with behavioral, neurochemical, or histopathologlcal techniques. A great deal has been written about SEPs and related procedures, and excellent sources exist (Cracco and Bodis-Wollner 1986; Regan 1989).

SEPs AND CLINICAL DISORDERS

The practical utility of SEPs in clinical medicine has long been recognized. Halliday and coworkers first demonstrated the usefulness of pattern reversal visual-evoked potentials (VEPs) in detecting clinical disorders, including optic neuritis (Halliday et al. 1972) and multiple sclerosis (Halliday et al. 1973). During episodes of visual impairment, VEPs recorded following stimulation of the affected eye were smaller in amplitude and prolonged in latency in comparison with periods of remission. Comparisons of VEP latencies recorded from normal subjects and patients with multiple sclerosis reveal clear differences (Bodis-Wollner et al. 1979; Halliday 1981). These and other successes led to widespread clinical use of SEPs. Some of the clinical conditions in which evoked potentials have been reported to be altered are presented in table 1. In studying clinical disorders, evoked potentials have been found to provide objective confirmation of other reports of sensory dysfunction, to be sensitive to both mild and severe sensory impairments, to aid in localization of lesions, to help in charting the course of diseases and treatments, and to be invaluable in studying patients with poor ability to communicate, such as infants and young children. Evoked potentials are used widely to monitor the status of sensory tracts during the course of neurosurgery (Jones 1986). These versatile tools have much to offer the neurotoxicologist.

SEPs AND NEUROTOXIC DISORDERS

Properties that make SEPs clinically useful also make them attractive for studying neurotoxicity. Over the past 15 years, a number of review articles have been written that serve to chronicle the development of the technology as well as the growing application to toxicological problems (Woolley 1977; Fox et al. 1982; Dyer 1982,1983,1985,1986,1987*a,* 1987*b;* Rebert 1983; Dyer and Boyes 1983; Arezzo et al. 1985; Otto et al. 1985, 1988; Otto 1986; Seppalainen 1988; Mattsson and Albee 1988; Mattsson et al. 1989*a,* 1990*a,* 1992; Boyes 1992). What began as a relatively limited and experimental application of the

TABLE 1. *Examples of neurological conditions in which SEPs are altered*

Condition	Reporting Source
Aging	Celesia and Daly 1977; Morrison and Reilly 1989; Simpson and Erwin 1983
Albinism	Creel et al. 1981; Apkarian and Spekreijse 1986
Alzheimer's disease	Coben et al. 1983; Smith et al. 1990
Amblyopia	Levi and Manny 1986
Amyotrophic lateral sclerosis	Cosi et al. 1984; Dasheiff et al. 1985; Bosch et al. 1985
Aphasia	Rothenberger et al. 1982
Asphyxia	Hrbek et al. 1977; Hecox and Cone 1981
Cochlea/auditory nerve damage	Don 1986
Blood-brain barrier disruption	Warnke et al. 1989
Cerebral lesions	Giblin 1964; Bodis-Wollner 1976; Nakanishi et al. 1978; Obeso et al. 1980; Chu 1986
Color blindness	Regan and Spekreijse 1974
Coma	Starr and Achor 1975; Starr 1976; de la Torre et al. 1978; Hume et al. 1979; de la Torre 1981; Karnaze et al. 1982; Stohr et al. 1987; Facco et al. 1988
Dementia	Abbruzzese et al. 1984; Goodin et al. 1978; Michalewski et al. 1986
Diabetes	Trick 1991
Down's syndrome	Bigum et al. 1970
Epilepsy	Ratliff and Zemon 1984
Glaucoma	Atkin et al. 1983; Bodis-Wollner 1989
Guillain-Barre	Brown and Feasby 1984
Head trauma	Greenberg et al. 1977; Shaw 1986; Jabbari et al. 1987; Mahapatra and Bhatia 1989

TABLE 1. *(continued)*

Condition	Reporting Source
Human immunodeficiency virus	Koralnik et al. 1990
Huntington's disease	Noth et al. 1984; Josiassen et al. 1982
Hydrocephalus	McSherry et al. 1982; Onofrj et al, 1981
Hyperactivity	Halliday et al. 1984
Ischemia	Mizrahi and Crawford 1984
Learning disability	Duffy 1986; 0llo and Squires 1986
Leukodystrophy	Markand et al. 1982; Garg et al. 1983
Migraine	Nyrke et al. 1989
Minamata disease	Mukuno et al. 1984
Multiple sclerosis	Halliday et al. 1973; Cant et al. 1978; Dorfman et al. 1978; Nuwer and Namerow 1981; Barajas 1982; Bottcher and Trojaborg 1982; Matthews and Wattam-Bell 1982; Matthews and Small 1983; Oishi et al. 1985; Toyonaga 1988; Sartucci et al. 1989; Ghilardi et al. 1991
Parkinson's disease	Mintz et al. 1981; Bodis-Wollner et al. 1981; Hansch et al. 1982; Bodis-Wollner et al. 1986
Phenylketonuria	Giovannini et al. 1988
Optic neuritis	Halliday et al. 1972
Optic tract lesions	Onofrj et al. 19826; Sherman 1986
Retinopathy	Papakostopoulos et al. 1984
Reye syndrome	Goff et al. 1983
Spinal cord injury	Perot 1973
Syringomyelia	Anderson et al. 1986
Tumors	Takayasu et al. 1987
Vascular lesions	Noel and Desmedt 1975

techniques grew steadily, utilizing more sophisticated methodology and providing more frequent and broader applications. Now it is possible to incorporate evoked potential testing into toxicological testing protocols (Mattsson et al. 1992). Guidelines have been drafted for conducting visual-, auditory-, and somatosensory-evoked potential procedures in testing the potential toxicity of compounds regulated by the U.S. Environmental Protection Agency under the Toxic Substances Control Act (1976) or the Federal Insecticide, Fungicide, and Rodenticide Act (1988 amendment) (Boyes 1990). If such guidelines are adopted, the routine use of SEPs in studying compounds suspected of damaging sensory systems is likely to continue growing.

There are many interesting examples of toxic damage produced to sensory systems that have been studied using evoked potentials. For example, ototoxicity is produced by exposure to several different solvents, including toluene, xylene, carbon disulfide, methylene chloride, and styrene in addition to trichloroethylene (Pryor et al. 1983, 1984*a,* 1984*b,* 1987; Rebert et al. 1983,1989 *a,* 1989*b,* 1991; Rebert and Becker 1986). Rats exposed to trichloroethylene for 12 weeks showed dose-related amplitude deficits in brain stem auditory-evoked responses (BAER) that were more severe at 16 kHz than at 8 or 4 kHz (figure 2). These permanent, frequency-dependent changes are indicative of hearing loss and are thought to result from damage to cochlear hair cells. The auditory deficit is clearly evident in the amplitude/intensity functions presented in figure 2. Clinically, it is probably more common to use BAER latency/intensity functions in studying auditory dysfunction because amplitudes are somewhat more variable across subjects. BAER latency/intensity functions were shown to be sensitive to alterations produced by developmental exposure to alcohol (Church 1987), and both latency and amplitude/intensity functions were altered by prenatal cocaine exposure (Church and Overbeck 1990). Finally, the systematic reduction of BAER stimulus levels, such as would be required to generate amplitude or latency/intensity functions, can be used to estimate auditory thresholds. These procedures have been shown to correlate well with behaviorally determined changes in auditory thresholds and histologically measured loss of cochlear hair cells produced by the ototoxicant trimethyltin (Crofton et al. 1990).

Somatosensory deficits induced by neurotoxic compounds have also been studied using SEPs (Dyer 1987*b).* For example, acrylamide-induced changes in somatosensory-evoked potentials have been reported in rats (Boyes and Cooper 1981) (figure 3) and monkeys (Arezzo et al. 1981) (figure 4). Acrylamide produced progressive latency increases and amplitude reductions of somatosensory-evoked potentials in both species. The evoked potential changes in monkeys preceded both behavioral and neuropathological signs of intoxication. Thus, somatosensory-evoked potentials can be sensitive

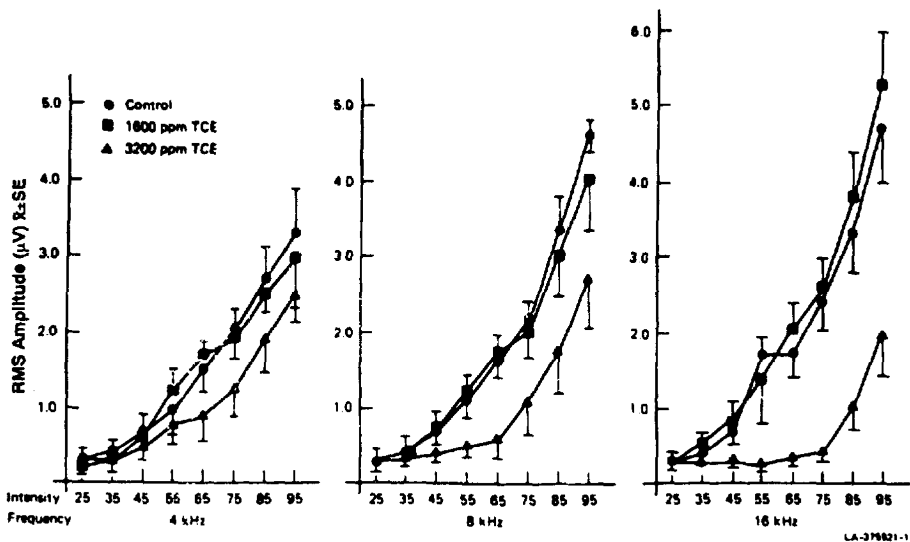


FIGURE 2. *Trichloroethylene-induced reductions in amplitude of tone-elicited BAERs*

SOURCE: Reprinted with permission from *Neurobehavioral Toxicology and Teratology* 13(1), Rebert, C.S.; Day, V.L.; Matteucci, M.J.; and Pryor, G.T. Sensory-evoked potentials in rats chronically exposed to trichloroethylene: Predominant auditory dysfunction. Copyright 1991, Pergamon Press plc. (Elmsford, NY).

indicators of central/peripheral distal axonopathy. Somatosensory-evoked potentials are typically elicited with electrical shock stimuli applied to a selected peripheral nerve through either skin or needle electrodes. Such stimuli bypass the sensory receptors that normally transduce stimulus properties such as pressure, vibration, or temperature into sensory neural activity. Evoked potentials recorded under these conditions provide somewhat limited information regarding sensory perception. It is likely that techniques under development involving more natural skin stimulation such as brushing or vibration (Pratt and Starr 1986) will correlate better with perception and also find useful toxicological applications. VEPs have been used to study many known or potential neurotoxic compounds. Boyes (1992) lists some of the compounds studied following acute exposures, Virtually every compound tested that was previously thought to be neurotoxic was found to produce changes in VEPs at some dose level. These studies suggest that VEPs might be useful in the detection phase of neurotoxicity investigations. However, because of practical constraints involved in screening many compounds to detect neurotoxicity, VEPs will likely be more useful in

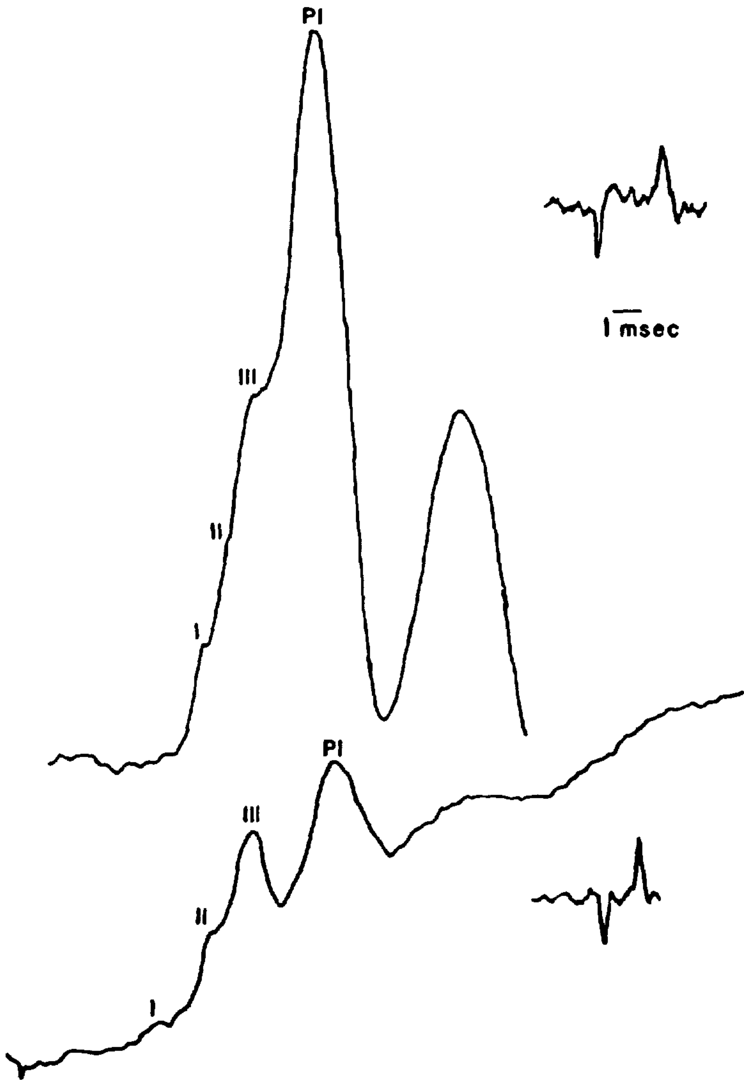


FIGURE 3. *Acrylamide-induced reduction in amplitude of far-field somatosensory-evoked potentials in rats*

SOURCE: Reprinted with permission from *Neurobehavioral Toxicology and* Teratology, 3, Boyes, W.K., and Cooper, G.P. Acrylamide neurotoxicity: Effects on far-field somatosensory evoked potentials in rats. Copyright 1981, Pergamon Press plc. (Elmsford, NY).

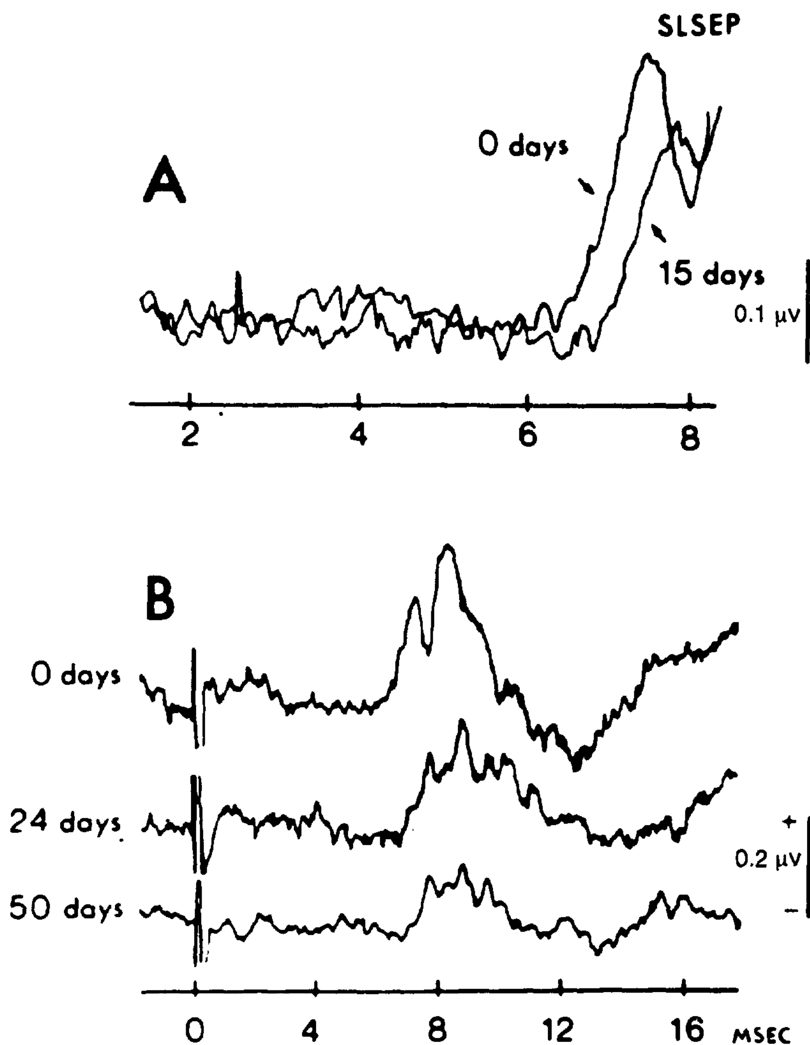


FIGURE 4. *Acrylamide-induced changes in amplitude and latency of far-field somatosensory-evoked potentials in monkeys. SLSEP-short latency SEP.*

SOURCE: Reprinted with permission from *Neurobehavioral Toxicology and Teratology,* 7(4), Arezzo, J.C.; Simson, R.; and Brennan, N.E. Evoked potentials in the assessment of neurotoxicology in humans. Copyright 1985, Pergamon Press plc. (Elmsford, NY).

characterization of changes in the visual system once abnormalities are discovered. Effects on evoked potentials with complementary data from other procedures can reveal much regarding the nature of the visual dysfunction produced. For example, reports that developmental lead exposure produced scotopic visual deficits in behavioral studies (Bushnell et al. 1977) prompted a series of investigations into the nature of lead-induced visual deficits by Fox and coworkers. VEP deficits (Fox et al. 1977, 1979; Fox 1984), in conjunction with other data, including electroretinograms (ERGs), prompted the discovery of selective action of lead on rod, but not cone, photoreceptors (Fox and Sillman 1979; Fox and Farber 1988; Fox and Chu 1988). In another example, VEP deficits, again along with changes in ERG, helped demonstrate the development of visual toxicity in metabolically altered rats treated with methanol (Lee 1987; Eells 1991). Finally, increased latencies and decreased peak-to-peak amplitudes of VEPs recorded from both visual cortex and the optic tract following exposure to trimethyltin indicated that this well-known limbic-system toxicant also produced retinal damage (Dyer et al. 1982), a supposition subsequently confirmed histologically (Chang and Dyer 1983).

SEPs AND MORE SOPHISTICATED QUESTIONS

Given that SEPs aid in the detection and characterization of neurotoxicity, what do changes in evoked potentials mean? The answer to this question takes several directions, two of which are considered here: (1) What is the cellular locus or pharmacological nature of the action of the compound, and (2) what are the perceptual correlates of the effects (i.e., how well can the subject hear, see, or feel)? The first answer leads to investigations of the mechanism of action of intoxication. To provide the second answer, it is necessary to study the relationship between evoked potentials and sensory psychophysics.

SEPs and Mechanisms of Action

Evoked potentials, as neuronal population responses, provide a broad level of analysis and therefore are not generally considered for use in mechanism of action studies. However, there is nothing to prevent the design of studies using evoked potentials that can help elucidate possible mechanisms of action. When used in conjunction with other techniques, they can be powerful.

Several authors have shown that acute and chronic exposure to toluene can produce a variety of effects on SEPs in rodents and humans (Dyer et al. 1984, 1988; Rebert et al. 1989*c;* Urban and Lukäs 1990) in addition to the ototoxicity discussed previously (Pryor et al. 1983, 1984*a,* 1984*b;* Rebert et al. 1983, 1989*a).* Mattsson and colleagues (1989*b)* demonstrated that acute inhalation of high concentrations of toluene vapors, as might occur during human toluene

abuse, produced an unusual episode of high frequency oscillations overriding normal rat SEPs (figure 5). EEG power spectra were also altered in a fashion that, along with the SEP changes, suggested the excitatory phase of the drug-induced users' "high." The spectra showed that this set of changes also could be produced by the toluene metabolite *o*-cresol, but not by other major toluene metaboliteS. These results suggested that the *o*-cresol metabolic pathway of toluene could be of importance in sustaining drug abuse behavior. It is unknown whether the neurotoxicity that follows repeated exposure to high concentrations of toluene, and that in a simulated abuse paradigm produced changes in flash-, auditory-, and somatosensory-evoked potentials and caudal nerve action potentials, is likewise attributable to *o*-cresol (Mattsson et al. 1990*b*).

Another example illustrating the use of evoked potential technology to address more mechanistic scientific questions is a set of studies regarding the effects of chlordimeform, a member of the formamidine class of insecticides and acaricides. Chlordimeform has an unusually selective effect on rat VEPs.

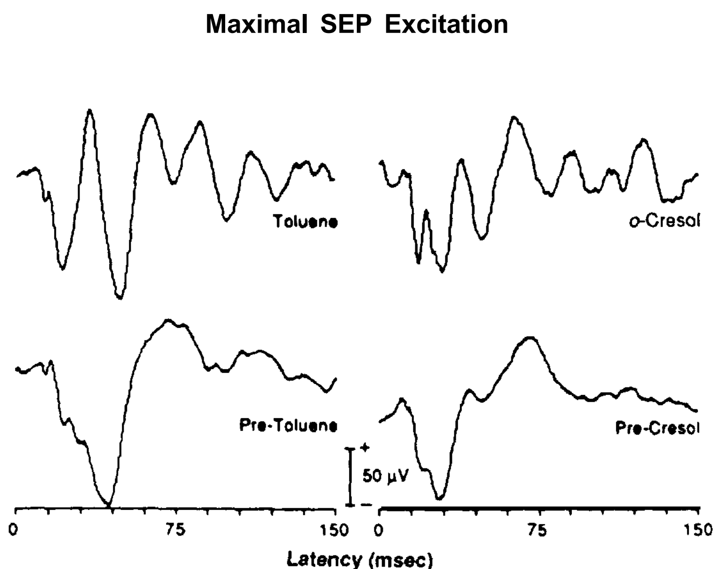


FIGURE 5. *Toluene and o-cresol-induced osillations in rat somatosensory-evoked potentials*

SOURCE: Reprinted with permission from *Neurotoxicology and* Teratology, 11(1), Mattsson, J.L.; Albee, R.R.; and Gorzinski, S.J., "Similarities of toluene and *o*-cresol neuroexcitation in rats." Copyright 1989, Pergamon Press plc. (Elmsford, NY).

When reversing black-and-white patterns were used as stimuli to elicit pattern reversal-evoked potentials (PREPs), the response amplitude was increased many times by acute treatment with chlordimeform (Boyes and Dyer 1984). However, the amplitude of flash-evoked potentials (FEPs) recorded from the same rats was unchanged. Amitraz, another formamidine pesticide, had similar acute effects on VEPs, but the duration of the effects was more prolonged (Boyes and Moser 1987). In contrast to the selective effects on amplitudes, peak latencies of both PREPs and FEPs responses were increased. In subsequent studies it was shown that the latency increases were a secondary consequence of chiordimeform-induced hypothermia. However, the selective amplitude effects could not be attributed to body temperature changes (Boyes et al. 1985*c),* nor could they be attributed to monoamine oxidase inhibition (Boyes et al. 1985*a),* at one time a leading candidate for the mechanism of pesticidal properties of the formamidines (Aziz and Knowles 1973; Beeman and Matsumura 1973).

This left two leading hypotheses regarding the possible mechanism of action of chlordimeform in altering rat VEPs. One was that the effects could have been attributed to local anesthetic-like actions (Chinn et al. 1977; Lund et al. 1978; Pfister et al. 1978). Recently, the formamidines have been shown to enhance susceptibility to amygdaloid kindling through local anesthetic-like properties (Gilbert and Mack 1989). However, the author and colleagues were unable to mimic the VEP effects of chlordimeform across a range of doses of lidocaine (unpublished observation), so this mechanism of action did not appear to be relevant to the visual effects of the formamidines.

An alternative hypothesis was suggested from reports that chlordimeform, and more potently its demethyl and di-demethyl metabolites, were agonists of octopamine, a neurotransmitter in firefly light organs (Hollingworth and Murdock 1980) and locust neuromuscular junctions (Evans and Gee 1980). Insect octopamine receptors are pharmacologically similar to mammalian α_2-adrenoceptors, suggesting that chlordimeform might act as a mammalian α_2-agonist (Hollingworth and Lund 1982). Yohimbine, an α_2-antagonist, attenuates other toxic manifestations of formamidines (Moser and MacPhail 1985), and some actions of the formamidines had been demonstrated on mammalian α_2-adrenergic systems, largely in the peripheral nervous system (Hsu and Kakuk 1984; Hsu and Lu 1984; Hsu and McNeel 1985; Hsu and Hopper 1986; Hsu et al. 1986). In VEP studies, rats treated with clonidine, an α_2-agonist, showed PREP amplitude augmentations that were very similar to those produced by chlordimeform (Boyes and Moser 1988). Pretreatment with yohimbine, which was not effective given alone, was able to block the effects of subsequent treatment with either clonidine or chlordimeform (figure 6). These results suggested that the actions of chlordimeform in the rat visual system may

AVERAGED EVOKED POTENTIAL

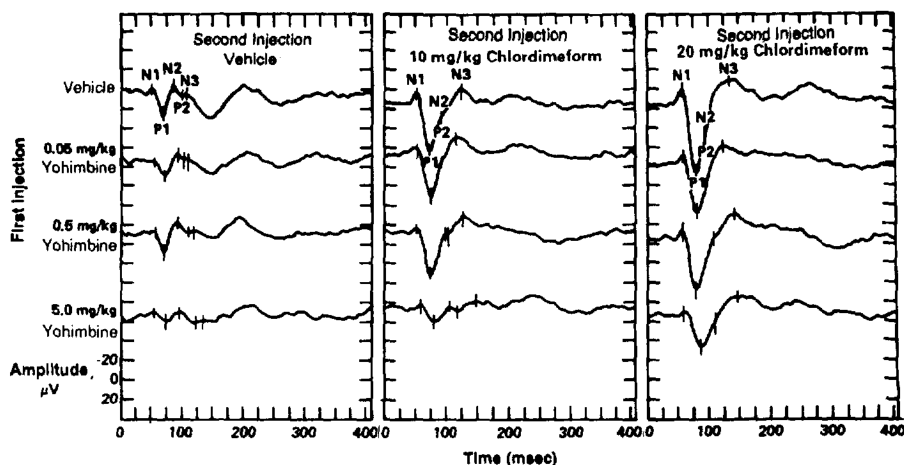


FIGURE 6. *Chlordimeform-induced increases in amplitude of PREPs in rats, which are blocked by pretreatment with yohimbine*

SOURCE: Boyes and Moser 1988

involve agonist-like properties at α_2-adrenoceptors, Interestingly, the actions of amitraz on dentate gyrus field potentials following perforant path stimulation, to increase hippocampal excitability and reduce tonic inhibition, also are mimicked by clonidine (Gilbert and Dyer 1988). Costa and coworkers have demonstrated in a series of studies that metabolites of chlordimeform, as well as amitraz, bind potently to α_2-adrenoceptors in the mammalian central nervous system (CNS) (Costa and Murphy 1987; Costa et al. 1988, 1989*a*,1989*b*). Thus, the VEP studies in conjunction with other reports helped demonstrate a mechanism of action of the formamidine insecticides in the mammalian CNS.

SEPs and Sensory Function

Answering the question "What do changes in evoked potentials mean?" also involves the relationship between changes in SEPs and changes in sensory function. When there are changes in evoked potentials, does that mean that the ability to see, hear, or feel is altered, and if so, in what fashion? In general, when there are changes in evoked potentials, changes in sensory capacities are expected, but the fidelity of the relationship between evoked potential changes and sensation depends on a number of factors.

Perhaps the most important of these factors is the sensory stimuli used to study sensory function and elicit evoked potentials. One basic concept in sensory physiology is that within sensory modalities there exist parallel processing channels, sometimes involving anatomically distinct cells or regions of the brain (Campbell and Robson 1968; Sachs et al. 1971; Campbell 1980; Lennie 1980; Regan 1982; Stone and Dreher 1982; Livingstone and Hubel 1988; Lennie et al. 1990). Each of these channels is responsible for encoding distinct stimulus parameters, such as visual color or motion, sensation of vibration or pain, or hearing high as opposed to low pitch. Thus, it is reasonable to expect that selective deficits within a sensory modality can be produced by exposure to neurotoxic compounds.

If evoked potentials are to be compared with other measures of sensory function, it is important to use the same stimulus conditions. Most commonly, sensory function is examined using behavioral procedures referred to as sensory psychophysics (Evans 1982; Moody and Stebbins 1982; Maurissen 1988). When evoked potentials and sensory psychophysics have been studied in the same subjects and using the same sensory stimuli, the two procedures have shown remarkably similar results. Campbell and Maffei (1970) and Campbell and Kulikowski (1972) measured VEPs to stimulus patterns for which the stimulus contrast (the difference in luminance between the bright and dark parts of the pattern, adjusted for the mean luminance) varied. They showed that the amplitude of the evoked potentials was a linear function of log contrast, and when the perceptual contrast thresholds were measured, they corresponded very closely with the contrast levels that failed to elicit evoked potentials. Although there are some exceptions (Regan 1989), the general conclusion of studies carefully comparing evoked potentials and sensory perception is that there is a close correspondence between the measures (Campbell and Maffai 1970; Campbell and Kulikowski 1972; Bodis-Wollner et al. 1972: Cannon 1983; Bobak et al. 1984, 1988). In laboratory animal studies, and in some human cases, SEP measures can be more readily performed than sensory psychophysics, because in part of the extensive time required to train animals to perform stimulus discrimination tasks. In addition, in human studies the need to communicate with and elicit the cooperation of subjects can be problematic in certain situations. For these reasons, SEPs have become an accepted tool for both the experimental study of sensory psychophysics and clinical tests of sensory function. An indication of the wide applicability and acceptance of evoked potential measures of sensory function is that a variety of measures of visual resolution, including visual acuity and visual contrast sensitivity, have been estimated using evoked potentials in many species, including goldfish (Regan et al. 1975) rats (Meyer and Salinsky 1977; Onofrj et al. 1982*a;* Boyes and Dyer 1983; Fox 1984; Harnois et al. 1984; Silveira et al. 1987), opossum (Silveira et al. 1982; Picanco-Diniz et al. 1983), rabbits (Kulikowski 1978; Pak

1984) ground squirrels (Jacobs et al. 1980), cats (Berkley and Watkins 1971, 1973; Campbell et al. 1973; Freeman and Marg 1975; Harris 1978), dogs (Odom et al. 1983), and nonhuman primates (Padmos et al. 1973; Previc 1986; Bonds et al. 1987). In humans, infants (Pirchio et al. 1978; Harris et al. 1976; Spekreijse 1978; Atkinson et al. 1979; Fiorentini et al. 1980; Sokol et al. 1983), healthy adults (Campbell and Maffei 1970; Campbell and Kulikowski 1972), and clinical patients (Steele et al. 1989) also have been studied.

The close correspondence between evoked potential and psychophysical results means that evoked potential measurements can be used to provide an indication of the nature of the deficits in sensory perception. For example, electrophysiologically determined audiograms have been used to study the toxicity of adult exposure to trimethyltin (Crofton et al. 1990; Fechter and Carlisle 1990) and developmental exposure to monosodium glutamate (Janssen et al. 1991).

Another example involves a return to the study of chlordimeform discussed previously, in which chlordimeform increased the amplitude of PREPS, but not FEPs (Boyes and Dyer 1984) through agonist-like actions at α_2-adrenoceptors (Boyes and Moser 1988). However, this mechanism of action tells us nothing regarding the nature of the visual changes following chlordimeform exposure or why PREPS were affected but not FEPs. To help answer these questions, Boyes and colleagues (1985*c)* investigated a number of stimulus manipulations in conjunction with treatment with chlordimeform, including stimulus luminance, pattern size, orientation, and contrast. The effects of chlordimeform were clearly dependent on stimulus contrast, appearing only for high-contrast stimuli. This explained the lack of effects on FEP amplitudes, because the FEP-eliciting stimuli, bright flashes, were devoid of contrast. This result implies that α_2-adrenergic receptors are involved somehow in regulating the neural encoding of stimulus contrast. Saying what this means for visual perception is still a difficult proposition, but one would expect differences in the perception of high-contrast patterns.

The issue of evoked potential studies of sensory function should not be left without noting that evoked potentials provide information regarding behavioral states or cognitive processes, in addition to sensory information. For example, a negative wave in the FEP of rats, occurring at about 160 ms (N_{160}), is influenced by a number of factors that are independent of the sensory stimulus per se, such as the number of days tested (Dyer 1989; Herr et al. 1991*a*). This peak, which is actually the first portion of a rhythmic flash evoked after discharge, appears to reflect behavioral sensitization and habituation-like processes. These extrasensory correlates of evoked potentials also can be exploited profitably in neurotoxicity studies, such as those showing (N_{160} to

be reduced in amplitude following exposure to solvents, including toluene and xylene (Dyer et al. 1988), carbon disulfide (Herr et al. 1992*a),* and others (Herr et al. 1991*b,* 1992*b).*

ANIMAL-TO-HUMAN EXTRAPOLATION

The procedures for recording SEPs from laboratory animals and from human subjects can be superficially quite similar (figure 1). The stimuli may be either identical or scaled for the relative sensory capacities of the species being studied. The electrodes, although affixed differently, should record electrical activity from analogous portions of the sensory systems, to the extent that the systems of different species have analogous components. Creel and colleagues (1974) and Mattsson and coworkers (1989*a)* have presented evoked potentials that are generally similar in a number of species. The extrapolation of toxicological data from one species to another is a major source of uncertainty in assessing exposure risks (National Research Council 1983). Therefore, if SEPs are to be recorded from laboratory animals and used to make decisions regarding the potential for human neurotoxicity, then a systematic comparison of the evoked potential responses among species is warranted.

A strategy for comparing the responses among different species is needed. Perhaps the strongest comparison would be to show that a variety of neurotoxic compounds produced corresponding changes in laboratory animals and humans and with similar dose-response relationships. Making this comparison is not possible because of the ethical problems in deliberately delivering known neurotoxic compounds to human subjects. Occasionally, human cases of inadvertent overexposure to neurotoxic compounds are discovered, but the sporadic nature of the occurrence of the cases, the unknown dose levels, and the presence of confounding variables make systematic comparisons with animal studies impractical, if not impossible. A more available approach consists of parametric stimulus manipulations, drug treatments, and studying cases of human neurodegenerative diseases.

The first part of the strategy involves recording evoked potentials from laboratory animals and healthy human volunteers and comparing changes in responses to manipulations of stimulus variables such as pattern size and rate of stimulus presentation (Hudnell et al. 1990*a;* Hudnell and Boyes 1991). The rationale for these comparisons is that if the sensory systems of the different species operate similarly (i.e., if they show comparable changes in response to the stimulus manipulations), then it follows that they may respond similarly to toxic compounds that reach the target tissue.

In parametric comparisons of human and rat VEPs, the stimuli used were light and dark vertical bars in which the luminance varied in a sinusoidal pattern across the face of the stimulus screen. These so called sine-wave gratings are commonly used in visual research, and the pattern size, that is, the width of the bars, is expressed as spatial frequency or the number of cycles of the stimulus pattern per degree of visual angle. Spatial frequency is to be distinguished from temporal frequency, which refers to the rate at which the stimulus is modulated over time, expressed in Hz. When the temporal rate is less than approximately 3 Hz, the individual "transient" evoked potentials are visible, and data analysis typically involves measuring the amplitude and latency of individual components in the response. With faster stimulus rates, the response takes on "steady-state," sinusoidal appearance in which the primary temporal frequencies of the response are driven by the stimulus rate. Steady-state responses are typically analyzed using Fourier-transformation-based spectral analyses, and data are expressed as phase and either spectral power or spectral amplitude of response components at various frequencies, Of particular interest is the response amplitude at the stimulus rate and harmonics. The temporal modulation can take several different forms, including pattern reversal, in which the light and dark portions of the pattern alternate positions, and pattern appearance/disappearance, in which the stimulus pattern alternates with a nonpatterned, blank screen of the same mean luminance as the stimulus pattern. Note that in either pattern reversal or pattern appearance/disappearance, there is no overall change in stimulus luminance, so the recorded potentials reflect aspects of the neural encoding of the change in stimulus pattern.

Pattern-evoked potentials recorded using either transient or steady-state temporal modulation have a number of similar properties in humans and rats. For example, the transient pattern-evoked potential peak labeled N110 in figure 1 showed a selective reduction of amplitude after pattern adaptation in both species (Hudnell et al. 1990*a,* 1990*b).* The spectral amplitudes of steady-state pattern appearance/disappearance VEPs recorded under comparable conditions from rats and humans are presented in figure 7. The pattern sizes were smaller in humans (higher stimulus spatial frequency), which reflects the ability of humans to resolve finer visual details than rats, but otherwise the stimuli were similar. The responses were recorded from extradural skull electrodes chronically implanted in rats, but necessarily from the scalp of humans. The closer proximity of the recording electrodes to the cortical tissue generating the potentials accounts for the higher voltage responses recorded from rats than from humans. In response to manipulations of pattern size (spatial frequency), the 1F component (the response activity occurring at the stimulus rate) in both rats and humans showed a "bandpass" spatial frequency profile in which amplitudes were greatest at intermediate spatial frequencies. In both species, the peak and overall shape of the 1F amplitude curves resembled

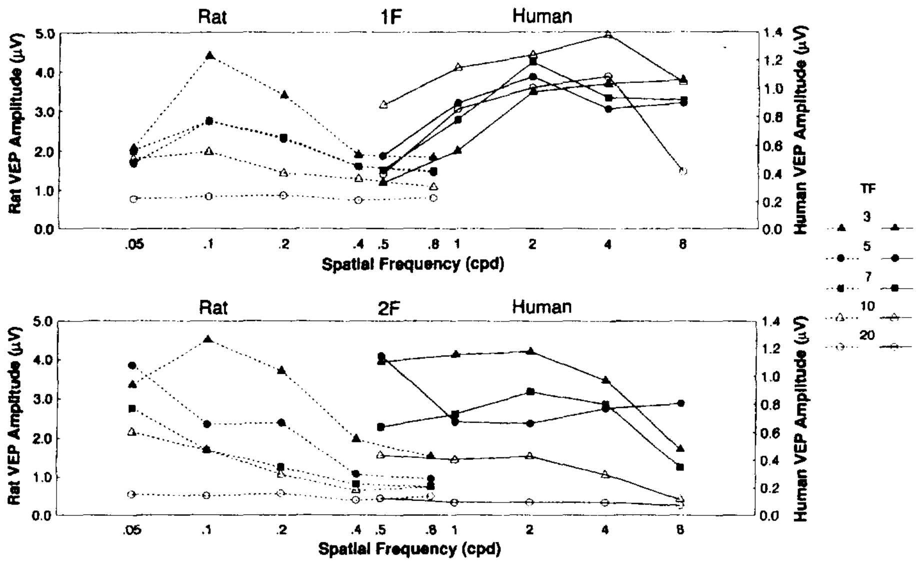


FIGURE 7. *Rat and human spectral amplitudes following on-off pattern modulation at the stimulus frequency (1F) and first harmonic (2F), as a function of pattern size (spatial frequency) and stimulation fate (temporal frequency [TF])*

SOURCE: Hudnell and Boyes 1991

the peak and shape of the respective contrast sensitivity function curves of the two species (Campbell and Robson 1968; Birch and Jacobs 1979). However, the 1F curves of the human and rat subjects did appear to differ in response to temporal frequency manipulations. The response profiles of the two species were rather similar at low temporal frequencies, but differed as the stimulus rate increased, with humans better able to follow high stimulus rates. In contrast, the 2F curves of both species were similar in both spatial and temporal domains. However, the 2F spatial profiles differed from the 1F profiles in that the responses tended to be largest at low spatial frequencies. Also, in contrast to the 1F data that differed in temporal response across species, as temporal frequency increased, the 2F responses of both rats and humans declined at a similar rate. Thus, stimulus manipulations revealed both similarities and differences between the rat and human response profiles. It follows that some aspects of human spatial vision, as reflected in the pattern VEPs, may be adequately modeled in laboratory rats to be predictive of human neurotoxic susceptibilities, but others, such as the response to high temporal stimulation rates, may not be predictive.

In the second phase of this strategy, the responses to neuroactive drugs of laboratory animals and human volunteers are compared. If the responses to drugs are similar, then perhaps the responses to other, less benign, exogenous compounds also will be similar. Human volunteers and rats were given diazepam, an agonist at the benzodiazepine-binding site on the gamma-aminobutyric acid (GABA)-receptor complex, and pattern appearance/disappearance VEPs were recorded. Both species showed an amplitude reduction of 2F at intermediate spatial frequencies but no significant changes in 1F amplitude (Hudnell and Boyes 1991). These results suggest a similar role for GABA-ergic modulation of the pattern-elicited responses in the rat and human visual systems and imply that other compounds altering the homeostasis in this system may act similarly in the two species. Although the larger implications of findings such as these to other neurotoxicants acting on other neurotransmitter systems or at nontransmitter sites are unknown, the more that two systems in different species can be shown to operate on similar principles, the more likely it is that neurotoxicants acting on unknown mechanisms would produce similar results in the two species.

The third part of the experimental strategy, still under way, involves comparing the responses to known neurodegenerative diseases in humans with animal models of the human conditions. If a disease such as generalized epilepsy results in SEP changes (Ratliff and Zemon 1984) and similar effects can be quantified in animal models of epilepsy, then greater confidence is warranted that degenerative conditions caused by neurotoxicants also may be expressed similarly in evoked potentials of laboratory animals and humans,

The research program on recording human and rat evoked potential data using comparable procedures has also led to cross-species mapping, which involves the development of a mathematical equation to predict the value of a dependent variable in one species from data derived in another species (Benignus et al. 1991). This process was applied to some of the rat and human VEP data presented in figure 7, namely the 1F amplitude values across spatial frequency, recorded at a temporal frequency of 3 Hz. First, a mathematical function relating amplitude to spatial frequency was fitted to the data from each species, and 95-percent confidence limits were calculated (figure 8). Next an equation was derived, the cross-species map, that describes the relationship between the functions for each species. In this case, the cross-species map was a linear function (figure 9), actually two linear functions, reflecting the prediction of the rising and falling portions of the human curve from the corresponding and noncorresponding limbs of the rat function. The fact that both curves are linear is a consequence of fitting both rat and human raw data using a single, symmetrical function, differing only in the parameters of fit. Perhaps functions

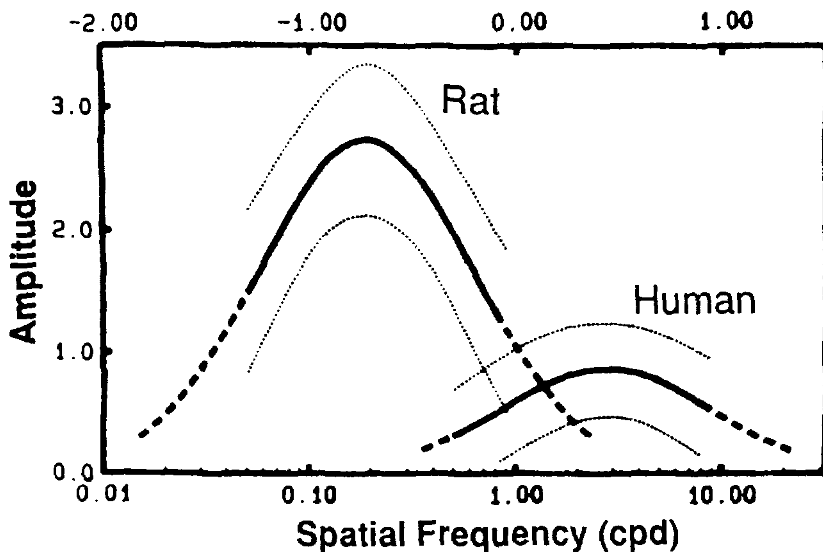


FIGURE 8. *Tuning curves and 95-percent confidence bounds fit to the rat and human data at a 3-Hz stimulation rate from figure 7.*

SOURCE: Benignus et al. 1991

such as these will someday be useful in attempting to predict potential human neurotoxicity from laboratory animal data.

In summary, these approaches to cross-species comparisons give a measure of confidence that most neurotoxic changes found in rats will be predictive of similar changes in humans. It would be prudent to expect that sole reliance on a species such as the rat will open the possibility of missing some relevant sensory toxicity because rats do not possess all the sensory capacity, such as color vision, present in humans. The rate of false negatives, perhaps the most important category of errors in toxicity testing and risk assessment, cannot be assessed from these types of investigations. In addition, these comparisons do not take into account factors such as metabolic differences, which may produce cross-species differences in toxicity, but focus only on comparability of the measured endpoints. However, for both economic and ethical reasons, rodents likely will be the mainstay of routine toxicity testing for some time. These types of cross-species comparisons do address the relevance of the rat data for estimating human risk. Our results to date suggest a good degree of comparability between pattern-evoked potentials in rats and humans, suggesting that neurotoxicity data collected in rats using these procedures may be predictive,

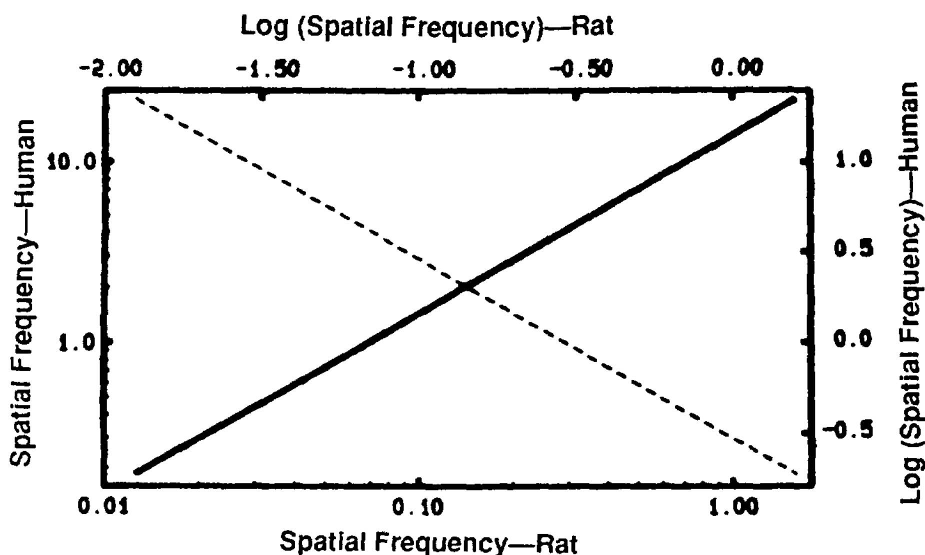


FIGURE 9. *Equations for mapping rat spatial frequencies as equivalent human spatial frequencies*

SOURCE: Benignus et al. 1991

with some exceptions, of similar changes in humans. In other words, the false-positive rate may be relatively low. The comparability of evoked potential measures across species in combination with the good relationship between evoked potential changes and changes in sensory function, discussed above, lead to the conclusion that changes in SEPs produced in laboratory animals following exposure to potential neurotoxic compounds may be predictive of neurotoxicant-induced deficits in sensory function in humans.

CONCLUSIONS

There is a need to incorporate tests of sensory function in laboratory animal toxicity testing. It is clear that sensory dysfunction may frequently occur, but go undetected, in standard animal toxicological testing protocols. SEP technology can be employed to address this need, can provide information regarding perceptual deficits arising from toxic insults, and can yield information regarding possible mechanisms of action. In addition, SEP changes in laboratory animals can be predictive of sensory changes in humans. Further research is needed to better define the neural substrates of evoked potentials and their perceptual correlates so that the implications of evoked potential changes for the cellular

targets, mechanisms of actions, and perceptual correlates of evoked potential changes can be better understood. More extensive use of these tools in animal toxicology is needed to better understand the ability of this methodology to detect sensory changes and to predict human toxicity from animal data.

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DISCUSSION

Q: Do you expect these neurophysiological or neuroanatomical changes?

A: Yes, they occurred in the monkeys. In the monkeys there was a degeneration of the retinal ganglion cells, and I would expect that is what we would see in the rats as well.

Q (Landfield): I would imagine one of the major control problems that you have is the arousal state of the animal. Arousal can have dramatic effects on the size of the evoked potential. It can cut it in half.

A: That is an issue that we have to deal with, particularly when some of the neuroactive compounds can have effects on arousal level, stress, etc. It comes out in a variety of ways: pupil size and body temperature. Body temperature, particularly in rats, can be strongly affected by exposure to toxic compounds. And the latency to EPs [evoked potentials] can be a linear relationship to body temperature. So you have to model that. We have been looking at the later waves of the EPs. The ones that I have talked about today are pretty early,

and they are thought to be closely tied to the sensory phenomenon itself. But there are later latency potentials which are thought to be more closely related to arousal, habituation, and sensitization-like phenomena. Those potentials actually can be very sensitive to neurotoxic compounds, particularly the solvents. We have a peak occurring at about 160 milliseconds after a flash-evoked potential which has been altered in a variety of solvent exposures, like toluene, xylene, carbon disulfide-a whole series of those. We are in the middle of a research program right now that tries to tie those changes to what we know about the behavioral effects. It is something that we need to consider, but it is also something that we can use.

Q (Landfield): I was just thinking that you could be lesioning the reticular of the arousal system, thinking you are affecting the visual system. Even the initial primary sensory-evoked potential is affected by arousal.

A: Yes, you are right. In our tests, our animals are harnessed (and we test them without anesthetic because that may interfere with the neurotoxic compound that we are testing), and the restraint itself can be stressful; and I think that may equate them all at a high level of arousal. It is not a good answer. The other aspect to your question-having lesions elsewhere and detecting the effects in the visual system-is for us a mixed blessing because, when we are testing unknown compounds, we want to know if they are effective. That is the first question, And if you see effects on evoked potentials, you can subsequently do a research program to trace that down. But at least we have detected it. The fact that they may be dependent and interdependent on other systems can be seen as a mixed blessing.

Q (Tilson): When you are testing evoked potentials in a screen situation, you wouldn’t be looking at just one, you would probably be looking at several endpoints.

A: Yes, you would be looking at visual, auditory, and somatosensory combined together. And if you see effects on all of those, then you may suspect that it is a more general phenomenon. That is a good point.

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Silver Staining as a Tool for Neurotoxic Assessment

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INTRODUCTION

Neurotoxicology and neurotoxicity have become matters of great public concern, and efforts have intensified to find reliable and convenient methods to identify those chemicals and substances that are potentially harmful to health because of their toxic effect on the central nervous system (CNS). A reliable answer is needed for the following question: Have the basic units of the CNS (i.e., the neurons) suffered irreversible damage as a result of the toxic effect? In other words, the task is to find a handy method to detect signs of degeneration in neurons and to determine the location and extent of such neuronal degeneration in the CNS. The reduced silver methods' have in the past been the most effective procedures for mapping experimentally induced neuronal degenerations, and the results demonstrated by modern silver methods indicate that they also have the qualities necessary to make them a powerful method for screening neuronal damage caused by various neurotoxic substances including drugs of abuse.

Since the early part of of this century, silver methods have been used with great success to stain both normal and pathologic components of nervous tissue (von Fajersztajn 1901; Bielschowsky 1904; Ramón y Cajal 1904). The ease with which various tissue components attract silver and the high-contrast microscopic images provided by metallic silver deposits are two of the most attractive features of silver impregnation.

Because the molecular basis for silver staining is still largely unknown, silver methods have generally been improved and refined by the empirical method and then modified by individual investigators to serve the requirements of a special line of research. As a result, many silver staining protocols have been developed through the years for various purposes, and the choice of method is rarely a foregone conclusion. This makes it especially difficult for researchers or histologists just beginning to use these procedures. Different methods and their various modifications often must be tried to obtain optimal results. Because tinkering with methods is time consuming, the tinker-and-toil process is often neglected, with the result that silver procedures are frequently inconsistent or

inadequate. Therefore, silver methods have the reputation of being capricious, or in the words of Wolman (1955), "their practical application represents more often an art than a science."

But the art of silver staining has continued to attract the attention of experimental neuroanatomists and even an occasional neurochemist, and some procedures have been refined to the point where they can demonstrate certain components of the tissue with a high degree of specificity and consistency. To appreciate the unique advantages that the silver methods can bring to the morphologic screening of neurotoxicity, it is useful to review some of the accomplishments made possible with the aid of silver methods.

THE NAUTA PERIOD: THE SILVER AGE OF NEURONAL TRACT TRACING

When the reduced silver methods were introduced at the turn of the century, they were used primarily for the study of normal axons and for visualizing various pathological conditions in the human brain. Several years elapsed before the first reports on the use of silver methods for the tracing of experimentally induced axon degeneration appeared in the literature (e.g., Rasdolsky 1923, 1925; Hoff 1932*a*, 1932*b*; Schimert[2] 1938, 1939; Szentágothai-Schimert 1941). However, the real breakthroughs in the use of silver methods for the tracing of Wallerian degeneration[3] (Waller 1850) came with the discovery of the Bielschowsky modifications by Glees (1946), Nauta (1950), and Nauta and Gygax (1951) for frozen sections, followed by the popular 'suppressive" Nauta-Gygax method (Nauta and Gygax 1954). In the 1954 method, Nauta and Gygax introduced a pretreatment of the sections in phosphomolybdic acid and potassium permanganate before the treatment in an aqueous silver nitrate solution followed by an ammoniacal silver solution and development in a formaldehyde solution acidified with citric acid (Nauta reducer). The pretreatment was important because it allowed the suppression of the staining of normal axons, which in turn greatly facilitated the selective visualization of degenerating axons. It was later shown that selective demonstration of degenerating axons and terminals could be achieved by simply treating the sections for a short time in ammoniacal silver solution followed by a reducer (Eager and Barnett 1964, 1966), particularly if the tissue had been stored in fixative for a long time (Loewy 1969). Nonetheless, the pretreatment introduced by Nauta and Gygax became the method of choice for suppressing the staining of normal fibers, and a number of laboratories around the world made use of various modifications of the Nauta-Gygax method, especially after having realized that the demonstration of the finest degenerating axon arborizations and boutons is often optimal with rather short survival times, usually 2 to 3 days following the experimental lesion.

The experimental tracing of pathways by the aid of the Nauta-Gygax method and many other silver methods (e.g., Fink and Heimer 1967; de Olmos 1969; Ebbesson and Rubinson 1969; Wiitanen 1969; Eager 1970; Kalaha-Brunst et al.

1974; Desclin and Escubi 1975) was in large part responsible for the dramatic expansion of neuroanatomical knowledge in the sixties and seventies. One in every four or five papers published in the Journal of Comparative Neurology or Brain Research in the early 1970s was based on silver staining of degenerating axons. Several examples could be mentioned in which the increased precision made possible by silver methods produced results that decisively changed the way the brain is perceived. One example from a key topic may be sufficient to illustrate this point. The first anatomical demonstration of the familiar ocular-dominance columns in the monkey striate cortex was made possible by the Fink-Heimer modification of the Nauta method in 1968, and the results were published in Nature the following year (Hubel and Wiesel 1969). It is also worth mentioning that the 1960s renaissance in comparative neuroanatomy of nonmammalian species was in large part fueled by students of the Nauta school (e.g., Ebbesson 1970; Lohman and Mentink 1972; Karten et al. 1973) and many others, who mastered and adapted the silver methods for the tracing of pathways in nonmammalian vertebrates.

The heyday of the silver staining epoch lasted from the early 1950s into the 1970s. During this time, silver staining was the only practical method available for the tracing of long pathways in the brain. The classic text titled "Contemporary Research Methods in Neuroanatomy" (Nauta and Ebbesson 1970) reflects the heavy reliance on silver methods at the time; more than one-third of the chapters pertain directly to silver methods used in the experimental tracing of degenerating pathways. These chapters did not introduce new silver methods; there was, rather, considerable discussion about how to interpret the results obtained by the experimental silver methods. Although it had been convincingly shown that the silver methods commonly used for the demonstration of so-called "terminal degeneration" were sensitive enough to identify a large number of degenerating boutons (Heimer and Peters 1968), one lingering problem concerned the question of sensitivity; another problem was related to the unavoidable damage of fibers passing through areas lesioned to induce degeneration. This made it difficult sometimes to identify the cells of origin for the pathway under study.

In regard to the problem of sensitivity, and with the benefit of hindsight, the introduction of the pretreatment of the sections in phosphomolybdic acid-potassium permanganate was a mixed blessing. The pretreatment allowed the suppression of normal fiber staining but also introduced the risk of suppressing argyrophilia in the most delicate degenerating fibers; too extensive pretreatment had the effect of suppressing all staining. The degree of pretreatment, although crucially important, was a subjective and somewhat arbitrary decision.

As for the unavoidable damage of fibers passing through the lesioned area, this shortcoming on the part of the experimental silver methods was probably one of the most important factors for the rapidly increasing popularity of the

autoradiographic tracing method (Lasek et al. 1968; Cowan et al. 1972; Edwards 1972). Many structures, especially in the deeper parts of the forebrain and brain stem, could be more easily analyzed with the autoradiographic technique than with methods based on Wallerian degeneration, and the number of publications using experimental silver methods declined precipitously in the late 1970s and early 1980s. However, the dwindling popularity of the silver methods was caused not only by the arrival of the autoradiographic method but also by the introduction of many other powerful tracer methods, including the horseradish peroxidase method (Kristensson et al. 1971; LaVail and LaVail 1972), the fluorescent tracer method (Kuypers et al. 1977; Van der Kooy et al. 1978), and the Phaseolus vulgaris leuco-agglutinin technique (Gerfen and Sawchenko 1984). Because of the successes of these newer tracer methods, the silver method was increasingly viewed as old fashioned in the context of experimental tracing of pathways, and in recent textbooks on tract-tracing methods (e.g., Heimer and Záborszky 1989; Björklund and Hökfelt 1990), not a single chapter was devoted specifically to experimental silver methods. However, as discussed in some detail below, the silver methods did not disappear from the field of experimental neuroanatomy. Silver methods still offer some unique advantages in particular tract-tracing experiments (e.g., Carlsen et al. 1982; Záborszky et al. 1985; Heimer et al. 1987); but more important in the context of this review is a growing awareness of the advantages that reduced silver methods can offer in other important areas of neuroscientific endeavor, including neurotoxicology and ischemia research.

As the conclusion of this short history of silver staining, it should be emphasized that the tracing of pathways by means of silver staining of degenerating axons and terminals during the third quarter of this century provided a firm anatomical foundation for the modern era of neurobiology. It is an ironic coincidence that as the close of the century nears and the “decade of the brain” is celebrated, the reduced silver methods seem poised to provide a further legacy to neuroscience.

APPLICATION OF SILVER METHODS FOR SELECTIVE DEMONSTRATION OF DEGENERATION REMAINS A LARGELY UNEXPLOITED OPPORTUNITY IN NEUROPATHOLOGY

Although reduced silver methods lost much of their attraction in experimental neuroanatomy when new tracer methods based on specific neuronal uptake and axoplasmic transport were introduced in the 1970s and 1980s some silver staining procedures remained the methods of choice for specific neuropathologic phenomena including neurofibrillary tangles and senile plaques. Reflecting the recent surge of interest in Alzheimer’s disease, several new protocols for the staining of neurofibrillary changes have been published during the last few years (e.g., Sevier and Munger 1965; Yamamoto and Hirano 1986; Campbell et al. 1987; Braak et al. 1988; Hedreen et al. 1988; Yamaguchi et al. 1990). These methods are modifications of the classic Bielschowsky method (e.g., Sevier and

Munger 1965; Yamamoto and Hirano 1986) or incorporate principles that were developed in the field of experimental neuroanatomy in more recent years (e.g., the method by Campbell et al. 1987; Braak et al. 1988; Hedreen et al. 1988).

For the neuropathologist, the usefulness of silver methods is by no means limited to the detection of neurofibrillary changes. As already indicated, practically every tissue component is potentially argyrophilic, and specific silver methods have been developed for the study of various nervous tissue components, One of the best-known examples of such specialized methods is the silver carbonate technique for microglia developed by Del Rio-Hortega (1919, 1932).

With the possible exception of the many silver procedures developed by Del Rio-Hortega for the study of glia cells and the various modifications of the Bielschowsky silver technique for the study of axons and neurofibrillary changes in Alzheimer's disease and related disorders, metallic silver techniques never have become part of the technical arsenal in laboratories of general neuropathology. This may be explained in part by silver procedures' reputation for being difficult and capricious and by the rather natural tendency to avoid the unfamiliar. Nevertheless, this perception is unfortunate considering the advantages that the reduced silver methods can offer to those interested in degeneration of neurons and their dendritic and axonal processes (e.g., Grant 1970; Leonard 1979).

A striking experimental example of the superiority of the silver degenerating methods vis-a-vis regular Nissl stains for detecting degenerating neurons can be seen in the olfactory cortex following olfactory bulb removal. Reports of chronic atrophic changes in olfactory cortex several weeks or months after lesions in the olfactory bulb or olfactory peduncle had occasionally appeared in the literature (e.g., Winkler 1918; Uyematsu 1921; Allison 1953). However, in spite of many earlier studies with conventional Nissl methods, transsynaptic induction of degeneration in olfactory cortex neurons as a result of an ipsilateral bulbectomy was detected only when sensitive silver methods were employed (Heimer 1968; Price 1976; Heimer and Kalil 1978; Carlsen et al. 1982). This especially striking illustration of the power of the silver method for screening neuronal degeneration is illustrated in a recent paper by Switzer (1991) who argues persuasively for the general utility of silver methods in assessing neurotoxicity.

If certain steps in the basic silver protocols are modified, silver ions can be induced to form metallic silver preferentially on mitochondria, boutons, myelin, or glia or on mesodermal elements in blood vessels. In spite of this versatility and, in some cases, exclusive sensitivity (e.g., degenerating boutons cannot in general be detected on the light microscopic level with any technique other than the silver method), it is apparent that a number of more specific immunohistochemical techniques (e.g., for glial fibrillary acidic protein) are more attractive to the neuropathologists than the reduced silver methods. The

reading of well-known reviews of neuropathological methodology (e.g., Duchen 1984; Okazaki 1989) confirms that the advantages of modern reduced silver methods have rarely been exploited in general neuropathology. Nonetheless, the identification of damaged pathways in the human brain must certainly be considered a desirable goal of a postmortem examination, and the reduced silver methods offer some unique advantages in this regard (e.g., Voogd et al. 1990). Contrary to common belief, some products of axonal degeneration can remain for several years after damage to the human brain (Grafe and Leonard 1981). Therefore, even if people survive for a considerable time following lesions in the CNS, postmortem silver staining of such brains could provide important signs for the existence of such lesions as well as answers regarding the effect on specific pathways. This approach could become a sensitive complement to the more traditional methods of histological evaluation in neuropathology.

SILVER IMPREGNATION IN THE 1980s: NEW METHODOLOGICAL DEVELOPMENTS

Although the silver methods have been used only occasionally in experimental neuroanatomy during the past decade, several developments have nevertheless taken place in regard to the procedures of silver impregnation. For instance, important and selective methods have been introduced by Gallyas and colleagues, and the cupric-silver method for degenerating neuronal elements (de Olmos 1969) has appeared in several new and increasingly more powerful versions during the past decade.[4]

The basic principle of silver staining was outlined many years ago by Liesegang (191 l), who suggested that reducing groups in the tissue transform ionic silver to ultramicroscopic “nuclei” of metallic silver, which are then enlarged by the deposition of additional silver to the point where they become visible in the light microscope (photographic development). In other words, the final result depends on the initial distribution of binding sites for silver ions. Several investigators have attempted to clarify the mechanisms involved and the conditions that determine the success and the specificity of the staining (e.g., Nauta and Gygax 1951; Samuel 1953*a*, 1953*b*: Peters 1955*a*, 1955*c*, 1955*c*; Wolman 1955; Evans and Hamlyn 1956; White 1960; Eager and Barnett 1964, 1966; Giolli 1965; Loewy 1969; Giolli and Karamanlidis 1978). As already indicated, the use of a suitable pretreatment (Nauta and Gygax 1954) to accentuate the difference between degenerating and normal fibers was an important step in modern silver methods including the many modifications of the Nauta-Gygax method, the various methods developed by Gallyas and collaborators, and the cupric-silver method by de Olmos.

During the 1970s and early 1980s, Gallyas (1982*a*, 1982*b*) conducted a series of experiments on the mechanisms of silver staining and identified some of the

factors that determine the formation of metallic silver. Besides consolidating the Liesegang theory, Gallyas and coworkers (1980*a*, 1980*b*, 1980*c*) identified conditions that favored the staining of either degenerating axons or degenerating terminals. The success of the method for demonstrating degenerating axons (Gallyas et al. 1980*b*) is due in large part to the use of a physical developer (a reducer containing silver ions) rather than the commonly used Nauta reducer (formaldehyde acidified with citric acid). The method of staining degenerating terminals (Gallyas et al. 1980*c*), on the other hand, resembles the Eager (1970) method in its simplicity: following an alkaline pretreatment and a short immersion in an ammoniacal silver solution, the development takes place in a Nauta reducer. Characterized by low background, high contrast, and good reproducibility, the Gallyas methods are also well suited for dark field illumination at low magnification as well as for quantitative evaluations (e.g., Wolff et al. 1981; Záborszky and Wolff 1982).

The ability to induce a Golgi-like staining of neurons in many parts of the brain was described by Switzer (1976), who used injections of puromycin, a metabolic cytotoxin, to traumatize neurons before staining them with the cupric-silver method. In more recent publications, Gallyas and collaborators have described a method for the Golgi-like demonstration of “dark” neurons consequent to various pathological conditions or artificially produced by postmortem traumatization (Gallyas et al. 1990; van den Pol and Gallyas 1990). The success of this method depends on the use of an esterification procedure, which in combination with the physical development produces a selective and easily controllable demonstration of traumatized neurons. The most remarkable feature of the method described by Gallyas and colleagues is how quickly (a few minutes) after trauma the affected neurons can be revealed.

The cupric-silver method for the tracing of degenerating pathways was developed several decades ago (de Olmos 1966), but the excellent quality of the results was overshadowed to some extent by a rather involved procedure, and the method was not widely applied in tract-tracing studies. Although a cupric-silver solution was used by Richardson in 1960 for the demonstration of autonomic nerves, de Olmos discovered by accident that cupric ions could be helpful in suppressing the staining of normal fibers in the CNS. This result is amply supported by more recent examination of metal ions for suppressing normal tissue argyrophilia (Gallyas and Wolff 1986). In contrast to the suppressive step in the Nauta-Gygax method, which has a tendency to interfere with the argyrophilia of both normal and degenerating fibers, the cupric-sulfate treatment seems to be less prone to suppress the argyrophilia of degenerating axons and terminals, The original cupric-silver method (de Olmos 1969) was later modified, first to improve the demonstration of terminal degeneration (de Olmos and Ingram 1972) and then for the demonstration of traumatized neurons (retrograde and transneuronal degeneration) by Carlsen and de Olmos (1981).

In contrast to the various Gallyas versions of the silver techniques, which demonstrate particular subsets of neuronal degeneration (i.e., axonal, terminal, or soma-dendritic degeneration), the cupric-silver method provides an overall view of these changes occurring in traumatized neurons.

It appears at first that the Gallyas methods and the cupric-silver method might represent two approaches to demonstrate the same phenomena. However, some significant difference may exist with regard to the morphological molecular substrate responsible for the result in the Gallyas methods and the cupric-silver method. One possible reflection of this difference is the ability to reliably impregnate specific parts of normal forebrain and brain stem when using the cupric-silver procedure (de Olmos 1969).

USE OF SILVER METHODS IN NEUROTOXIC RISK ASSESSMENT

With the growing realization that functions of the CNS can be irreversibly compromised by a rapidly increasing number of drugs and chemical substances, neurotoxic risk assessment has become a matter of great urgency. A review of the literature suggests that neurotoxicologists are rediscovering the silver methods as a means of finding answers to questions of neuronal damage. Reduced silver methods have been used to great advantage for demonstrating neuronal degeneration in response to a number of neurotoxins, including 6-hydroxydopamine (Hedreen and Chalmers 1972; Maler et al. 1973), methamphetamine and methylenedioxymethylamphetamine (Ricaurte et al. 1982; Commins and Seiden 1986; Commins et al. 1987; Jensen et al. 1990, 1991), 3-acetylpyridine (Balaban 1985), diazepam (Balaban et al. 1988*a*), trimethyltin (Balaban et al. 1988*b*; Whittington et al. 1989), organophosphorus (Tanaka and Bursian 1989), and glutamate (Janssen et al. 1991; Schweitzer et al. 1991). The neurotoxic effect of tetanus toxin (Illis and Mitchell 1970) and capsaicin (Jancsó and Király 1980; Ritter and Dinh 1988, 1991) as well as the neuronal degeneration caused by excitotoxic amino acids including kainic acid (Jarrard 1983; Ben Ari et al. 1981), ibotenic acid (Jarrard 1989), and quinolinic acid (de Olmos and Beltramino 1991) also have been successfully demonstrated by the aid of silver methods.

To demonstrate the high degree of sensitivity available through the use of modern silver methods, the authors prepared material from animals injected with trimethyltin (figure 1) and quinolinic acid (figures 2 and 3). In the case demonstrated in figure 1 (prepared in collaboration with Karl Jensen at the Health Effects Research Laboratory at the Environmental Protection Agency at Triangle Park, NC), rats were injected intraperitoneally with trimethyltin in a dose of 8 mg/kg body weight; the rats survived for 48 hours (figure 1, panels A and B) or 24 hours (figure 1, panel C) following the injection. Pronounced neuronal degeneration is evident, not only in hippocampus (figure 1, panels A and B) and proisocortex (figure 1) panel C), but also in many other structures

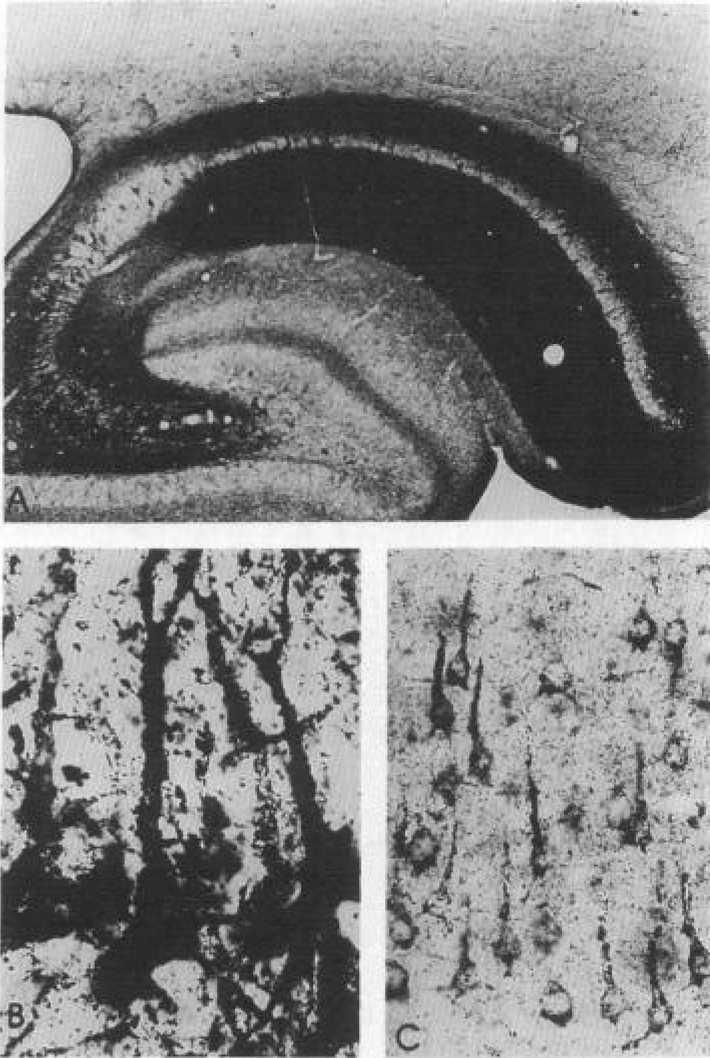

FIGURE 1. *A. Degeneration in the rat hippocampus following an intraperitoneal trimethyltin injection (8 mg/kg body weight) 48 hours before sacrifice of the animal. Note the large number of degenerating neurons, especially in CA3 and CA4, and the massive degeneration of terminals as reflected in the pitch-black areas in most of the hippocampus. B. Detail of some of the degenerating neurons in CA3. C. Degenerating neurons in rat insular cortex following a trimethyltin injection 24 hours before sacrifice of the animal (de Olmos' cupric-silver method).*

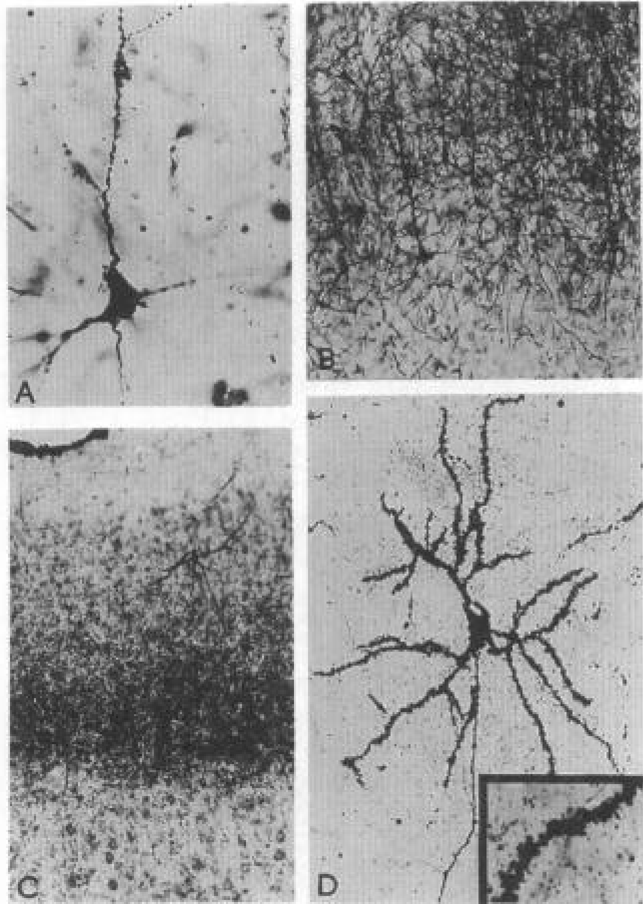

FIGURE 2. *Degeneration in parietal cortex of a rat in which quinolinic acid (60 nmol in 0.5 µL) was injected into the striarum (A, B, and D) or thalamus (C) 24 hours before sacrifice of the animal. The section in A was stained with Gallyas' methods for “dark” neurons, whereas those in B, C, and D were stained with de Olmos' cupric-silver method. Note the corkscrew appearance of the apical dendrite of the pyramidal cell in A and in many of the pyramidal cells in B (black arrows). The section in B was counterstained with neutral red. Like the neurons in A and B, the densely spined neuron in D shows signs of degeneration including an irregular cell body and beaded axon. The insert in D demonstrates the detailed rendition of dendritic spines from the dendrite indicated by the arrow. The heavy band of terminal degeneration in C is located in layers 3 and 4.*

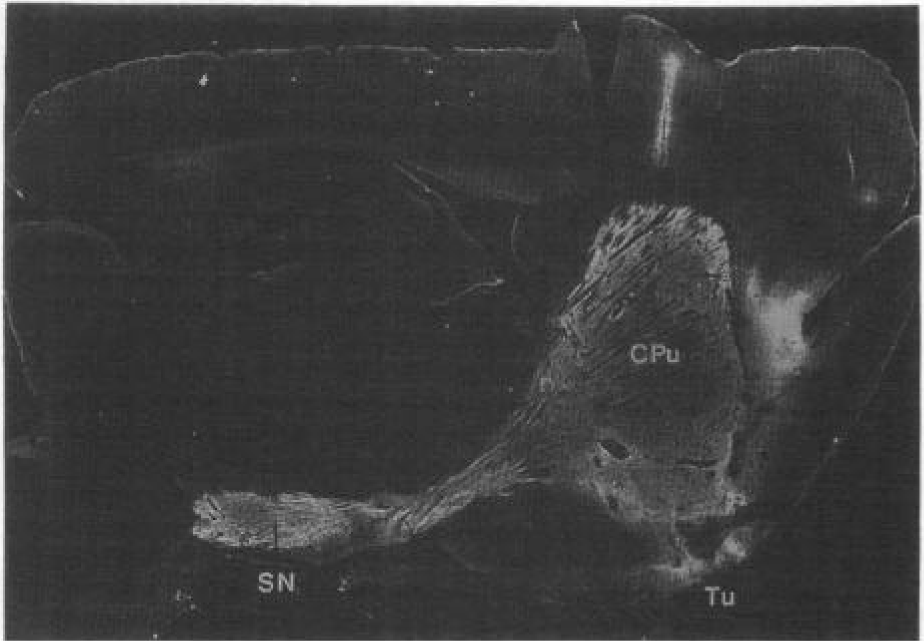

FIGURE 3. *Direct print of a sagittal section of a rat brain with a striatal injection of quinolinic acid (60 nmol/0.5 µL) 24 hours before sacrifice of the animal. The figure illustrates the high-contrast panoramic view of massive degeneration in the striatum, substantia nigra, and related pathways made possible by the silver method, in this case, the cupric-silver method.*

KEY: CPu=caudate-putamen; SN=substantia nigra; Tu=olfactory tubercle

throughout the forebrain and brain stem as described by Balaban and colleagues (1988*b*).

Figures 2 and 3 demonstrate degenerative neuronal changes in the rat brain following striatal or thalamic injections of quinolinic acid. The dose of quinolinic acid injected into striatum of the rats illustrated in these figures was 60 nmol in 0.5 µL. This is considerably less than the dose generally used in comparable experiments (e.g., Foster et al. 1983; Björklund et al. 1986; El-Defrawy et al. 1986; Foster and Schwarcz 1989; Beal et al. 1988; Boegman and Parent 1988; Calderon et al. 1988). Nonetheless, significant degeneration is manifest, not only in striatum, but in many distant areas, including the cerebral cortex (figure 2), endopiriform nucleus, claustrum, globus pallidus, septum, bed nucleus of stria terminalis, thalamus, substantia nigra, and several other brain-stem areas.

Although the low-magnification picture in figure 3 does not allow full appreciation of the neuronal degeneration in all these areas, it does give a synoptic view of the dramatic degeneration in dorsal and ventral striatum and closely related nigrostriatal and striatonigral fiber systems.

The advantages of the silver techniques may also be exploited in cases where brain tissue is damaged by hypoxic events (figure 4, panels A and B) or other forms of trauma (Crain et al. 1988; Franck and Roberts 1990; Freund et al. 1990*a*, 1990*b*; Lin et al. 1990; van den Pol and Gallyas 1990; Schmidt-Kastner and Freund 1991; de Olmos et al., in press). Figure 4, panel C, illustrates degenerating cell bodies in striatum following injection of the suicide transport agent volkensin (Wiley and Stirpe 1988) into the substantia nigra. Figure 4, panel D, demonstrates a Golgi-like staining of neurons traumatized by hypoglycemia. Degenerative changes can be demonstrated by the aid of the silver method as early as a few minutes following the insult (de Olmos et al., in press: van den Pol and Gallyas 1990).

One of the most attractive features of the silver method is its high degree of sensitivity. The reason for this resides in the capacity to initiate in situ silver deposits in response to favorable changes in the chemical groups of macromolecules.[5] Immunohistochemical techniques are also sensitive and able to detect early changes (Yanagihara 1990). In addition, these techniques are quite specific, but herein lies not only their strength *but also their weakness as potential general screening methods.* Furthermore, some of the immunoprocedures that are now being used in ischemia research (e.g., for microtubule-associated proteins and a-tubulin) are based on the disappearance of the immunoreaction in degenerating neurons and for that reason are less suited as screening methods in cases where a subpopulation of neurons is affected or in situations where degenerating neurons are isolated and scattered. On the other hand, if one knows where to look, a slight but detectible loss of immunoreaction for microtubule-associated protein can be appreciated in hippocampal dendrites after only 3 minutes of ischemia (Kitagawa et al. 1989).

Other immunohistochemical procedures can reveal specific proteins (e.g., heat-shock proteins) (Lindquist 1986) produced by the neurons in response to stress and trauma. Like the silver method, the immunoreaction of the heat-shock proteins can provide a positive Golgi-like image of traumatized neurons (Gonzalez et al. 1989). The affected neurons can be detected a few hours after the occurrence of the trauma, but the time that elapses between the synthesis of these de novo proteins and their disappearance is usually short and varies from one population of cells to another. Furthermore, it is generally presumed that only some immunoreactive cells die, whereas others may survive. The same comments are applicable in general to the recently discovered c-Fos protein and related immediate early gene products (Sagar et al. 1988; Morgan

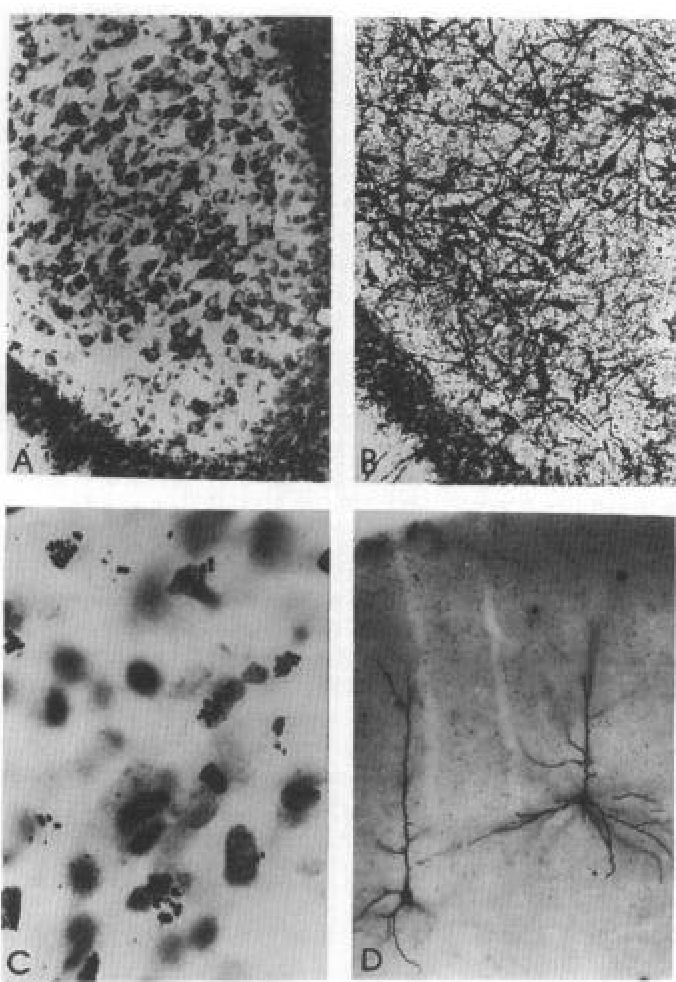

FIGURE 4. *A and B. Pictures from part of hippocampus in a gerbil subjected to 10 minutes bilateral occlusion of internal carotid arteries 6 hours before sacrifice of the animal. Whereas it is difficult to pinpoint the degenerating neurons in the Nissl stained section in A, the adjacent cupric-silver stained section shown in B leaves little doubt about the massive neuronal degeneration. C. Degenerating cell bodies in striatum of the rat following injection of volkensin (0.1 μL contains 3 ng) into substantia nigra 10 days before sacrifice of the animal (Gallyas' method for terminal degeneration). D. Degenerating neurons in the cerebral cortex of a hypoglycemic rat sacrificed 3 hours following intraperitoneal administration of insulin (25 IU/kg) (Gallyas' method for "dark" neurons).*

and Curran 1989), which have been used to localize the site of drug action (Robertson and Robertson 1989; Young et al. 1989; Graybiel et al. 1990).

It is interesting to note that some of the silver methods used in ongoing experimental studies of ischemia and other kinds of trauma (de Olmos et al., in press; van den Pol and Gallyas 1990) can produce positive evidence of neuronal injury at an earlier time following trauma than the heat-shock and the c-Fos proteins. Nonetheless, there are a number of immunohistochemical techniques that can now be applied to the study of neuronal activation and/or damage; the field is wide open and at an exciting stage, where much progress will be made by exploiting new interdisciplinary applications of various techniques.

From the experience gained so far, the silver techniques represent tools as sensitive as immunohistochemical methods for assessing neurotoxicity. The silver methods cannot indicate the neurochemical characteristics of the affected neurons, but they can provide a quick, sensitive, and comprehensive survey of neurodegenerative changes and direct subsequent searches for more specific pathometabolic changes. Furthermore, with the aid of silver methods, the extent of *irreversible* damage can be clearly assessed by direct observation of the degenerating neurons and their processes. Also, although silver is a precious metal, it is cheaper to buy the materials needed for silver staining than to purchase the often expensive antibodies needed for the immunohistochemical techniques. This additional advantage of using silver methods is a consideration during times of financial restraints or when screening neurotoxins on a large scale. However, in the final analysis, the reduced silver methods and the immunohistochemical methods, although comparable in sensitivity, are very different in many ways: both approaches have unique qualities that would seem to ensure their independent and complementary use in various aspects of neurotoxicology.

The advantage of using the silver method in the screening of neurotoxicity rests not only on its sensitivity and the high-contrast images it provides but also on the fact that it seems to be able to register all types of degeneration, and it does so in a pattern that demonstrates the morphology of the degenerating tissue elements. At present, it also may be unique in the ability to detect degenerating axons and boutons, especially when they are the principal site of action for a neurotoxin and when the parent cell body itself could survive the trauma. It is not generally appreciated that the silver technique may be adjustable for different conditions of fixation (e.g., both freshly and thoroughly fixed material). Although silver methods usually have been applied to frozen free-floating sections, the methods also have been modified for use on mounted cryostat sections of both fresh and fixed material (Hamlyn 1957; Hjorth-Simonsen 1970; Loots et al. 1977; Wheeler and Ritter 1984). Further developments of such modifications could facilitate the processing of a large amount of material in a routine fashion

and include the possibility of using adjacent unfixed sections with other neurohistological procedures.

As with most histotechnical methods, silver staining does present some initial hurdles for the newcomer. However, Switzer (1991) reminds researchers that, although small variations in one of the procedural steps in the silver staining can ruin the results, ". . . if the same diligence necessary for the success of other sensitive procedures, such as in biochemistry and immunocytochemistry, is applied to the silver methods, consistent results will be obtained." Interpreting the pattern of argyrophilia also may present an obstacle for a beginner, but this kind of uncertainty on the part of the inexperienced observer is not unique to the silver method; it is common to all histological stains. With regard to the silver methods, the problems of interpretation have been dealt with systematically by several authors (Heimer 1970; Giolli and Karamanlidis 1978; de Olmos et al. 1981). Furthermore, as in every other field of human endeavor, it is useful for the novice to profit from the guidance of more experienced individuals.

SUMMARY

There is no denying that the silver methods lost their dominant role as tract-tracing methods in the past 10 to 15 years. But it seems equally clear that the silver technique is headed for a dramatic revival in many fields of neuroscience, where the scope and localization of neuronal degeneration are a central issue. Together with the immunostaining of proteins formed or altered in traumatized neurons, the modern silver techniques provide neurotoxicologists and neuropathologists with unparalleled opportunities to detect and study injured and dying neurons. Characterized by great sensitivity and distinct rendition of the morphology of degenerating neurons and their processes, the reduced silver methods constitute the ideal tool for screening irreversible neuronal damage caused by neurotoxic substances including drugs of abuse.

Those interested in the rapidly expanding fields of "excitotoxicity" and neurodegenerative disorders (Taylor 1991) are also likely to find increasing use for the silver methods. The pattern of degeneration in so-called "system degenerations" may be predetermined by the neuronal connections (Saper et al. 1987), and as the disease progresses from the destruction of the originally affected neuron population, closely related systems and pathways may be recruited into the pathophysiologic cascade. Any type of trauma to the CNS has the potential to produce this type of "domino effect" of degeneration, through which additional systems are progressively recruited into a degenerative chain reaction of transneuronal degeneration. In other words, even longstanding disorders may exhibit signs of more recent degeneration, and the proper use of silver methods at autopsy may give some important clues regarding the etiology of disease: it may also provide new insights about the anatomy of the human brain.

Little can be said at present about the chemical basis of argyrophilia in degenerating and "reactive" neurons, but there is every reason to pay more attention to this subject. One can expect that a continuing and concerted effort will result in a rational understanding of the molecular biological and physicochemical events that fortuitously provide the basis for the selective impregnation of degenerating neuronal elements, This knowledge can be the basis for the development of even more reliable and simple, yet sensitive, silver methods suited for neurotoxic risk assessment on a large scale.

NOTES

1. If reduction of the silver ions into metallic silver is accomplished by the aid of a special reducing solution, the silver method is commonly referred to as a "reduced" silver method. Another main group of silver methods is represented by the various Golgi methods in which the reduction of the silver ions takes place without the help of a special reducing solution.

2. Until 1940 Dr. Szentgothai used his original family name, Schimert.

3. In 1850 Augustus Waller demonstrated that a nerve fiber that is separated from its cell body undergoes a gradual disintegration referred to as Wallerian degeneration. This important phenomenon forms the basis not only for the experimental silver methods but also for the famous Marchi method (Marchi and Algeri 1885) for the staining of degenerating myelin.

4. The cupric-silver method also has been introduced for the detection of proteins and peptides in gel electrophoresis (Switzer et al. 1979), and this principle is widely used by biochemists because of the high sensitivity in detecting amounts of protein in the nanogram to femtogram range.

5. Silver staining is based on a chemical reaction, the first phase of which is catalyzed by certain points of the tissue structure, whereas in the second phase the products of the first phase (submicroscopic metallic silver grains) serve as catalyst. For this reason, a favorable change in the composition of one single chemical group in a macromolecule, if it occurs within a potential catalytic point, is capable of initiating the in situ formation of a silver grain containing 10^3-10^8 atoms. This is why silver methods are sensitive enough to visualize disturbances undetectable by other kinds of histological methods, excluding the immunohistological methods, in case of which a change in one single chemical group generally results in the binding or deposition of only one molecule of the coloring agent.

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DISCUSSION

Q (Ricaurte): Have you come across the situation where a toxin can produce an insult and induce silver positivity as visualized by one of these methods, only to find later that it was a reversible change-that, in fact, a particular terminal or fiber or cell body did not degenerate, but there was just a reversible change in the cytoplasm?

A: I have not: I haven't really looked that much into neurotoxicology, until preparing for this talk.

A (O'Callaghan): I think George Kreutzberg at Max Planck spent several years studying a model of the facial nerve and looked at the cell-body reaction in facial nucleus, at microglial reaction, and at silver reactions in that cell body. The cell body does not degenerate. It gives you the classic cell-body reaction-silver degeneration pattern around the cell body-and you get a gliosis around the cell body. All that is reversible.

Q (Ricaurte): Is it a silver reaction of the cell body of origin?

A (Jensen): I think it is. I am not sure.

COMMENT (Ricaurte): At an early stage, degenerating cell bodies are highly silver positive. They are very easy to see. However, if someone comes along 4 weeks later using staining techniques and looks in the same area, they might conclude that there was no cell loss, when in fact, there is. This is difficult to detect. To show that argyrophilic is a reversible change, someone would need to go back and do a quantitative study and show that the same cell body that was silver positive was still present weeks later. Clearly, this would be a difficult and painstaking task. I always introduce the Fink-Heimer method as one of the few, if not only, anatomical techniques (at the light microscopic level) that can show fiber or terminal degeneration.

COMMENT (Heimer): I always thought that way.

Q (Seiden): Is the chemical basis for silver reaction in the dying nerve understood at all?

A: I do not think so. I am not a chemist, but I have read the many papers that have been written on the mechanism of silver staining. There was a series of papers published by Alan Peters, many years ago, and he suggested that there might be a protein, that is, an enzyme, that is argyrophilic. Maybe one way to think of it is that whatever the argyrophilic substance may be, much more of it becomes available to react with silver during the process of degeneration. In the various Nauta methods, potassium permanganate treatment is a key factor in the suppressive effect of argyrophilic. The argyrophilic-suppressing effect of permanganate may depend on oxidation of the argyrophilic protein. If you apply a little "suppression," you suppress the few available sites for protein-silver reaction in normal fibers; if you continue the suppression, you also eliminate the many sites that have become available in degenerating fibers, and with too long suppression, everything disappears.

Q (Cho): Are these cells permeable with silver? The silver really permeates these cells?

A: Yes.

Q (Cho): It distributes like water?

A: Yes. Everything is basically argyrophilic in the tissue. You can get silver into lysosomes and mitochondria.

Q: In the intact cell with no cell damage?

A: Absolutely.

Q (Molliver): A few years ago, Ray Lasek at Case Western reported that silver stained intermediate filaments. Is there any commonality with silver degeneration stains? Sternberger has several antibodies against phosphorylated intermediate filament proteins, and these accumulate in the damaged cell bodies. Have you looked at structures that stain with silver?

A: No, I haven't.

COMMENT (Molliver): The neurofilaments are synthesized in the cell body, transported into the axon, and are phosphorylated; but when axons are damaged, they accumulate in the cell body.

Q (Tilson): I have a couple of quick practical questions. As a neurotoxicologist, you have these changes in silver staining. How comfortable would you be in calling changes in silver staining following some treatment an adverse effect? Would you accept an increase in silver stain intensity as evidence of an adverse effect on the CNS?

A: I am not sure. I think we have to have more studies in this field, and I think that what is really missing are systematic studies of these processes at the electromicroscopic level. That might give us some answers in regard to what happens to these cells that take up silver so quickly. I think there are still many questions in need of answers.

Q (Tilson): If you saw no changes in silver staining, how comfortable would you feel in saying that that treatment is not neurotoxic?

A: No, I wouldn't be comfortable at all, because if there is one thing we neuroanatomists have learned the hard way, it is that you should never trust negative results. In short, that answers the question.

Q (Tilson): I have been told that traditional neuroanatomists believe that 20 percent of the cells in an area need to be affected before you can detect a change at the anatomical level. Do you agree with that?

A: Twenty percent of the cell population? No, I wouldn't say so. I think that you can see neurotoxicity even if only a few cells are affected, provided you have a good silver stain.

C (Tilson): I meant with the Nissl stain.

A: I agree with you. I think that half the cells in a given area can be affected, and you still can't see it.

Q (Tilson): Our testing guidelines for using hematoxylin and eosin and other stains would seem to be sensitive only to 20- to 50-percent changes. But with the silver staining, you say that the proportion of cells that would have to die to see a detectable change would be considerably less?

A: Oh, yes.

Q: What do you think the cupric method is adding to silver to enhance the sensitivity?

A: The addition of cupric salts improves the staining, probably by having a suppressive effect on normal tissue argyrophilic, thereby making the silver impregnation more manageable, as Dr. Gallyas and his coworkers have

suggested. Interestingly, Dr. de Olmos discovered the utility of cupric salts serendipitously; he dropped a beaker containing sections in a modified Bielschowsky silver solution on a warm copper plate. Eager to save the material, he collected the sections and the contaminated silver solution floating on the copper plate. Later he inspected the sections in the microscope, and, to his amazement, he discovered that the intensity and quality of the silver stain were dramatically improved. Dr. de Olmos quickly deduced that the contact of the silver solution with the copper plate was the key factor, and then it was only a matter of choosing the right cupric solution to repeat this result. The situation recalls Pasteur's famous line: "Where observation is concerned, chance favors only the prepared mind."

Q: After quinolinate, you can show the degeneration in the cortex after 15 minutes with cupric silver. Have you tried this with another stain?

A: I have not, but I believe Jose de Olmos has tried without much success.

Q (Landfield): Of course, the silver techniques are very valuable for detecting degeneration. But so many things in neurotoxicology and neuropathology require quantitative analysis, and I wonder if you would comment on what your feeling is about the application of these for a quantitative method or whether there is a potential for it.

A: Yes, that was why I showed the Gallyas technique. I think that with one or the other of Gallyas' techniques, one can direct to a certain point the silver to specific components of the neuron, for example, to degenerating axons or to the degenerating terminals. It can be done in a systematic fashion with great reproducibility, so that one can make a computer analysis of, for example, the density of degenerating boutons. One can follow it over time; it reaches a peak, and then it goes down. Although I don't have any experience myself, I think there is at least some potential with a technique like that.

Q: How about comparing animal to animal? I mean, that was within the same animal?

A: Yes, the pictures I showed were in the same animal. Well, again, we have not done it. I have to take their word for it. I think Gallyas and his collaborators mention this in their papers-even between animals.

COMMENT (Jensen): De Olmos' most recent modifications are extremely reproducible from animal to animal.

A (Heimer): By the way, de Olmos' recent modification is not published yet.

Q: You see silver degeneration in 15 minutes if you inject quinolinic acid in the striatum and look in the temporal cortex? What kind of mechanism would kill a cell that fast?

A: Good thought. I don't know-maybe from transneuronal excitotoxicity?

Q: I mean, axonal transport presumably couldn't get the compound back there that quickly. Do the cell bodies in the striatum following quinolinic acid injection? Do they take up the silver? In 15 minutes?

A: Yes, you have a central area which is relatively resistant to argyrophilic; but in the surrounding area, you do have argyrophilic within the striatum.

COMMENT (Olney): We studied kainic acid toxicity with the de Olmos method back around 1980 with Joe Price and Jim Schroll. In that case, I think we were studying seizure-mediated pathology, and so I hesitate to relate it directly to quinolinate toxicity, which is for the most part, I think, not seizure related. But we saw the same thing and in many brain regions following subcutaneous treatment with kainic acid. We saw neurons taking up the silver, showing their entire arborization, and looking beautiful-but rather sick, probably, some of it. But we had the same questions. Are these neurons whose cell bodies are taking up the silver, not just the axons? And if the cell bodies are taking it up, are they taking it up directly, or is the degenerative process starting at the cell body or at the axon; and is there a retrograde process here? We have no answer, but it was seizure-mediated pathology through many seizure circuits that we were studying.

Q (for Olney from Seiden): What do you think?

A (Olney): I don't know. I have looked at these kinds of lesions with electron microscopy and other methods as well as the silver. With striatal lesions, there is a degenerative reaction that you can see in the cortex, the deep layers of the cortex, primarily.

COMMENT (Heimer): Excuse me for interrupting. This is interesting because the degeneration we see is in the superficial layers.

COMMENT (Olney): It is a later degeneration that I am talking about in the deep layers. But, in addition, if you are talking about degeneration that occurs in the frontal cortex, for example, I think you mentioned; and if it is ipsilateral to the striatal lesion-that is, tissue that you have just been poking your needle through-there is reflux of the quinolinate up around the needle track and it gets into the cortex and spreads around up in the cortex, causing some direct excitotoxic damage in the cortex. That is something that I think could happen

in 15 minutes and could explain some degeneration in the cortex. But I don't know about the temporal cortex.

COMMENT (Heimer): Well, that is an important comment. These are the kinds of things that would be really important to study.

COMMENT (Olney): And quinolinate is also a peculiar excitotoxin. It has some characteristics of kainic acid and some of NMDA [N-methyl-D-aspartate]. The NMDA lesion tends to be very circumscribed to the area of injection. Kainic acid is spread all over the brain because it causes seizure pathology. All the pathways that interconnect the injection site with the rest of the brain transneuronally throughout the whole limbic system, in fact, all those systems get turned on with the propagating excitotoxic mechanism, in which glutamate is being released at many points throughout the brain. And quinolinate has some of that property, but not anywhere near as much as kainic acid.

Q (Seiden): In talking with Dr. de Olmos, I found that he could partially block some of the degeneration with, not MK801, but a different antagonist.

COMMENT (Olney): MK801 would do that. He didn't try it? He didn't try MK801?

COMMENT (Seiden): No, he didn't, he used one of the other. . .

COMMENT (Olney): MK801 would do that. And tomorrow I am going to show you that MK801 will also produce its own toxicity in the cortex, and there is a heat shock protein response to that which is very interesting in relation to the question about Fos proteins.

COMMENT (Jensen): He used ketamine.

COMMENT (Olney): Yes, ketamine and MK801 have the same mechanism.

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Mapping Toxicant-Induced Nervous System Damage With a Cupric Silver Stain: A Quantitative Analysis of Neural Degeneration Induced by 3,4-Methylenedioxymethamphetamine

Karl F. Jensen, Jeanene Olin, Najwa Haykal-Coates, James O'Callaghan, Diane B. Miller, and José S. de Olmos

INTRODUCTION

Evidence continues to accumulate implicating toxic substances in the etiology of a variety of neurological diseases. Consequently, the process of identifying, understanding, and regulating neurotoxic substances remains a pressing challenge. This challenge is complex because toxicants can injure the nervous system in a variety of ways. In addition, knowledge of the structure and function of the nervous system is not sufficient to entrust a single endpoint with predicting the neurotoxic potential of a substance. The purpose of structural assessments is to provide a convincing picture of the location and extent of damage to the nervous system. Silver stains that selectively reveal neural degeneration hold particular promise in this regard. Evidence that such histological procedures can selectively stain degenerating neurons has emerged from more than 25 years of anatomical tract tracing experimentation (Beltramino et al., this volume). In this chapter, the authors describe preliminary results using a recently developed cupric silver stain (de Olmos et al., in press) to delineate areas of the brain damaged by administration of 3,4-methylenedioxymethamphetamine (MDMA).

MDMA causes dramatic and long-lasting neurochemical alterations in the brain (Fuller 1989; Commins et al. 1987*a*; McKenna and Peroutka 1990; Schmidt 1987; Ricaurte et al. 1988; Scallet et al. 1988; Bittner et al. 1981; Schmidt and Taylor 1987; Stone et al. 1987; Davis et al. 1987; Schmidt et al. 1986). The extent to which MDMA-induced neurochemical alterations reflect neural degeneration remains controversial. To address this issue, the authors have mapped regions of the brain stained with the cupric silver method following administration of MDMA. Although a better understanding of the chemical

basis of the stain's selectivity for degenerating neurons is needed before the observed staining can be uniquely associated with particular degenerative events, the present results demonstrate that the cupric silver stain is a useful tool in determining the location and extent of structural alterations resulting from exposure to a toxicant.

ANALYSIS

MDMA (0, 25, 50, 150 mg/kg as salt) was administered to Long-Evans rats four times, with a 12-hour interdosing interval. There were four animals per dose group. The animals were housed individually in wire-bottom hanging cages (to prevent hyperthermia-induced mortality) and allowed food and water ad libitum. At various times after the last dose, animals were anesthetized with pentobarbital, perfused intracardially with saline followed by 4 percent paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). After perfusion, the brains were removed and a 4-mm sagittal block of the cerebrum was frozen and sectioned at 40 microns. All sections were collected and allocated to one of six sets. Sets of sections were stained with either cresyl violet or the cupric silver stain. Regions of silver staining unique to the treated animals were traced from four homologous sections from a 0.72 mm sample of the sagittal block and reconstructed using a computerized serial section reconstruction system. A survival time of 48 hours was employed for the dose-response analysis. For the time-course analysis, MDMA (100 mg/kg) was administered four times, with a 12-hour interdosing interval, except for animals in the group labeled "2x," in which MDMA (100 mg/kg) was administered only twice. Four animals were sacrificed at each survival time (18 hours, 48 hours, 60 hours, 7 days, and 14 days). An additional four animals were treated with fluoxetine (5 mg/kg) or MK-801 (1 mg/kg) 30 minutes prior to each of four doses of MDMA (100 mg/kg). Control groups included either fluoxetine or MK-801 or MDMA alone. Neurochemical evaluations of animals from corresponding cohorts are reported by O'Callaghan and Miller (this volume).

RESULTS

Evidence of cupric silver staining could be observed in several regions in control animals. Consistent and intense staining was apparent in the olfactory bulb. Diffuse and very light terminal-like staining was occasionally observed in the hippocampus. Scattered stained neurons occasionally could be seen in the forebrain. With the exception of the olfactory bulb and hippocampus, staining in control animals was sufficiently minimal to allow for a systematic comparison of different brain regions following administration of MDMA.

In animals treated with MDMA and sacrificed 48 hours later, the intensity of staining in the neocortex is dose related (figures 1 and 2). The staining is most prominent in the frontoparietal region of the neocortex. The majority of stained elements within the neocortex are axons and terminals. At lower doses (4x25 to

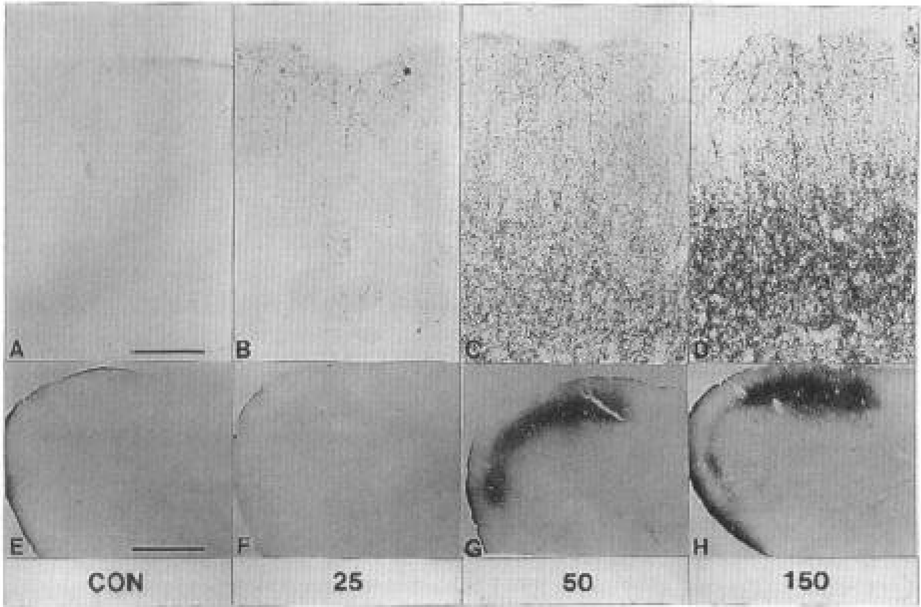

FIGURE 1. *Silver staining in sagittal sections of frontoparietal cortex in animals sacrificed 48 hours following various doses of MDMA. High magnification of upper neocorticel layers. Panels A through D: scale bar=100 mm. Lower magnification of frontoparietal cortex. Panels E through H: scale bar=1 mm. Control (panels A and E). four doses of 25 mg/kg given 12 hours apart over 2 days (panels B and F).*

4x50 mg/kg), staining is primarily in the upper layers, whereas at higher doses (4x75 to 4x150 mg/kg), it reaches deeper layers. Perikaryal staining is more pronounced at the higher doses.

Silver staining is not confined to the frontoparietal neocortex. In some cases, pronounced staining occurs in other brain regions, including the striatum and thalamus (figures 3 and 4). Such staining does not occur in all animals in any one dose group. For example, one animal receiving 4x75 mg/kg MDMA exhibited extensive staining within the striatum and thalamus (figure 3, panels A and D, and figure 4), whereas another animal receiving the same dose and exhibiting the same degree of damage within the neocortex did not exhibit staining in the striatum (figure 3, panels C and D). The predominant type of staining differed in the various brain regions. Although terminal, axonal, and perikaryal staining all were present in the neocortex, striatal staining appeared to be almost exclusively of terminals, and the thalamus exhibited staining of both axons and terminals.

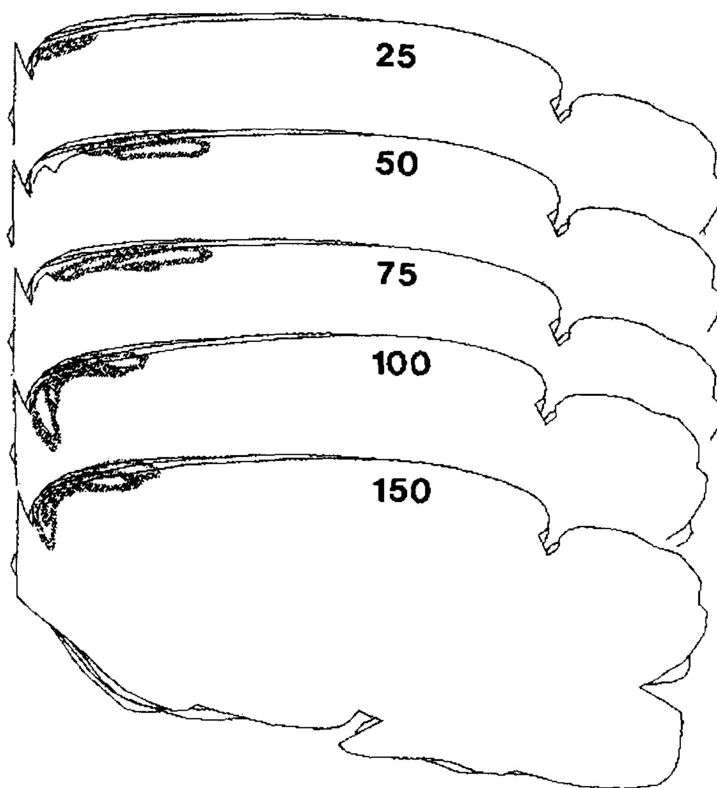

FIGURE 2. *Volumetric reconstructions of regions of intense silver staining (shaded areas) in the frontoparietal neocortex from four Sagittal sections after various doses in mg/kg of* MDMA.

Silver staining was apparent after the lowest dose (4x25 mg/kg), and the volume of staining in the neocortex and striatum was dose related (figure 5). The volume of degeneration was evaluated at various times after repeated doses of 100 mg/kg (figure 6). Substantial staining was evident as early as 18 hours after only two doses of 100 mg/kg. The extent of staining was only slightly smaller than that observed 16 hours after four doses of 100 mg/kg. Staining was maximal at 18 hours and declined thereafter but was still detectable at 14 days.

The effects of fluoxetine (5 mg/kg) and MK-801 (1 mg/kg) on MDMA-induced (4x100 mg/kg) silver staining in the frontoparietal cortex also were examined (figure 7). Neither fluoxetine nor MK-801 induced intense silver staining when administered alone Fluoxetine reduced by approximately half the volume of tissue stained and dramatically reduced the intensity of staining throughout the

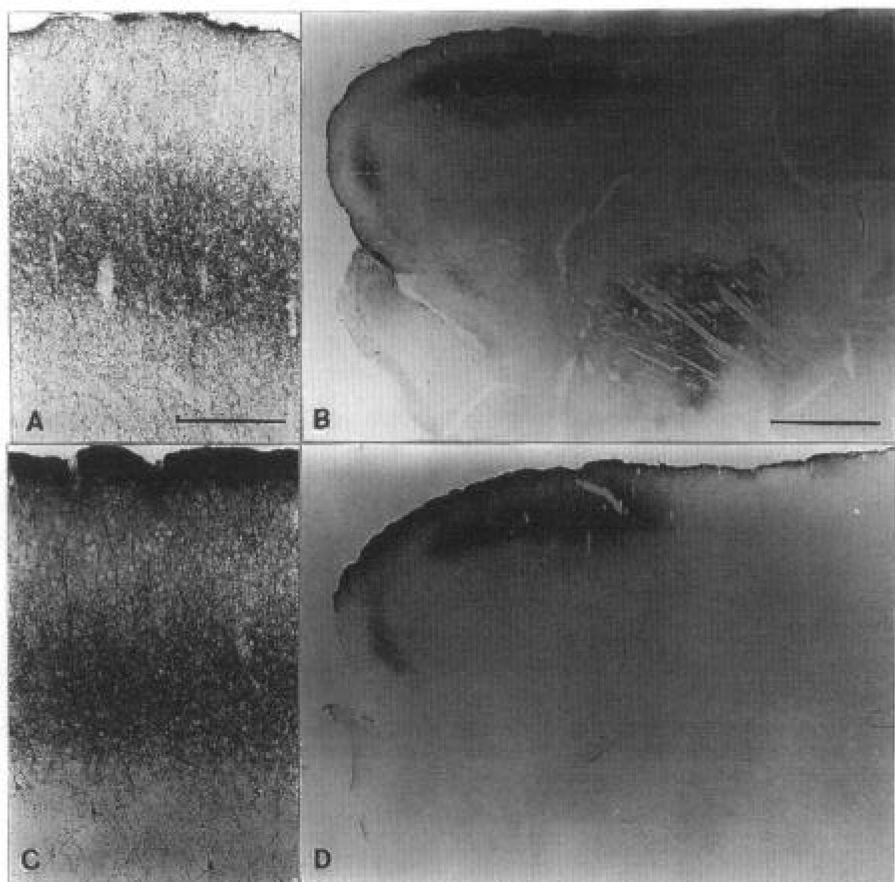

FIGURE 3. *Silver staining in the frontoparietal cortex and striatum 48 hours following the last dose of MDMA (4x75 mg/kg.) Panels A and B: sagittal section from an animal exhibiting damage to striatum. Panels C and D: sagittal section from an animal not exhibiting any silver staining in striatum. Panels A and C: high power of neocortex from sections shown in panels B and D. Note that the extent of silver staining in the neocortex is about the Same in both animals. Thus, it is unlikely that either variation in dosing or technical considerations pertaining to the histological method account for the differences. Scale bar=0.2 mm for panels A and C. Scale bar=1 mm for panels B and D.*

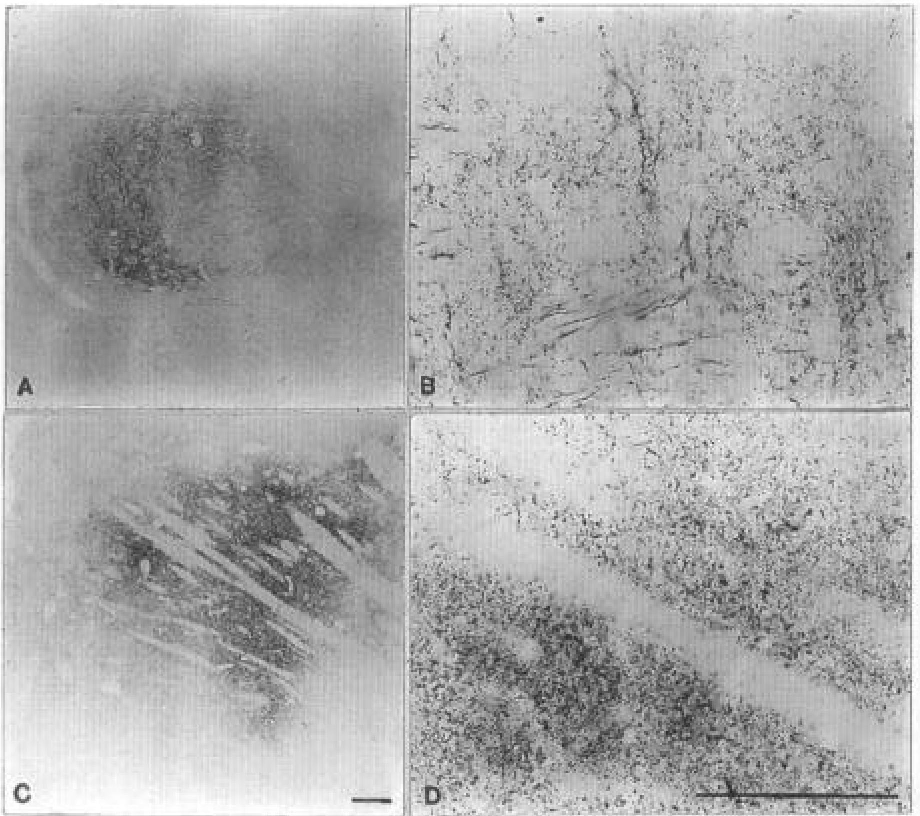

FIGURE 4. *Thalamic and striatal silver staining 48 hours after the fourth dose of MDMA (75 mg/kg). Panels A and B: section through thalamus showing both axonal and terminal degeneration. Panels C and D: section through striatum. From the same animal as in figure 3, panels A and B. Scale bar=0.2 mm for panels A and C. Scale bar=0.1 mm for panels B and D.*

affected regions. The reduction in intensity of staining was not associated with a preferential reduction in the staining of terminals, axons, and perikarya. In contrast to fluoxetine, MK-801 virtually eliminated evidence of MDMA-induced silver staining.

CONSIDERATIONS FOR THE INTERPRETATION OF SILVER STAINING

The authors' observations confirm a previous report (Commins et al. 1987*a*) that MDMA induces terminal, axonal, and perikaryal staining in the frontoparietal neocortex. We further assessed the volume of this staining within the neocortex

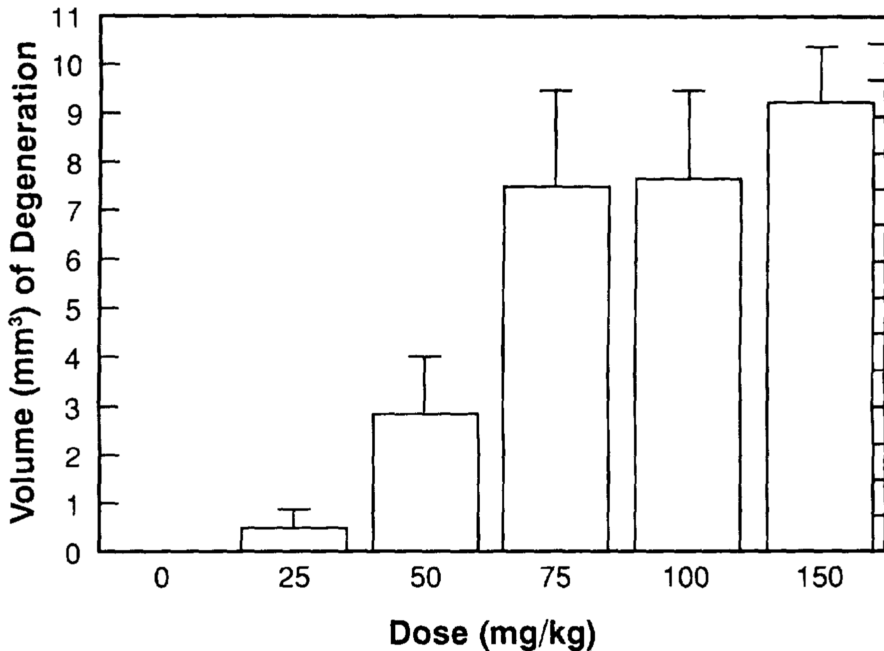

FIGURE 5. *Dose dependence of the volume of MDMA-induced neocortical and striatal silver staining. Each group included four animals. All animals received each dose of MDMA twice a day for 2 days (a total of four times). All animals were sacrificed 48 hours after the last dose. Standard errors indicated by vertical bars.*

and demonstrated its dose dependence. Despite the dramatic nature of this MDMA-induced silver staining, there are several considerations that need to be borne in mind when interpreting the present findings.

First, all doses of MDMA used in the present study produced intense silver staining. We therefore do not know a dose level at which MDMA produces no evidence of silver staining. The time-course data indicate that optimal staining occurs less than 48 hours following MDMA administration, and significant staining could be induced by only two doses of 100 mg/kg MDMA. Thus, when attempting to determine the highest dose of MDMA that does not induce silver staining, a survival time shorter than 48 hours may be more appropriate. In addition, a single rather than a multiple, dose also may provide a better estimate of the highest dose that does not induce silver staining.

Second, the chemical basis for the stain is not currently known. Consequently, silver staining cannot be specifically associated with particular degenerative

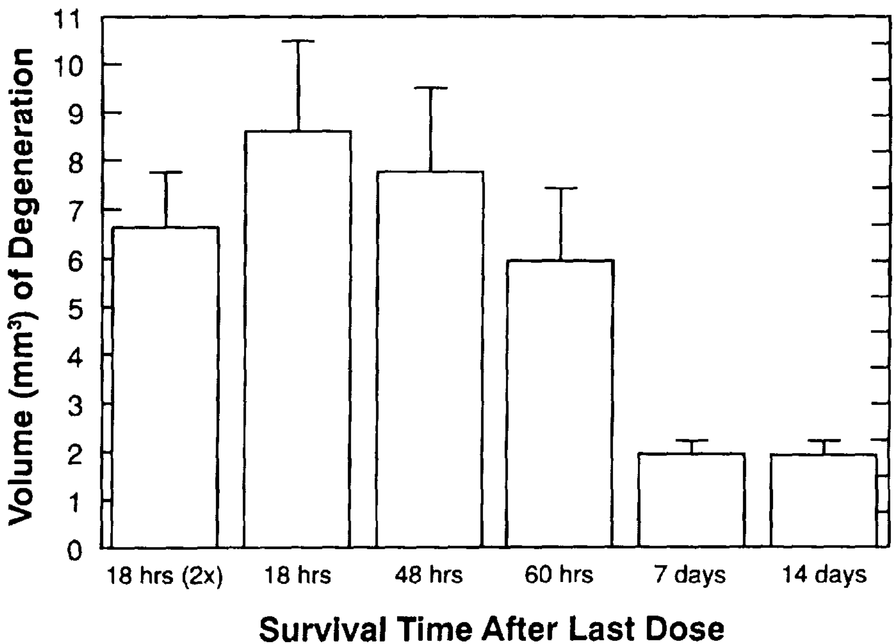

FIGURE 6. *Time course of the changes in the volume of MDMA-induced neocortical and striatal silver staining. All animals recieved 4x 100 mg/kg MDMA and were sacrificed at various times after the last dose except those in the group marked "2x," which received only 2x100 mg/kg MDMA and were sacrificed 18 hours after the second dose. Each group included four animals. Standard errors indicated by vertical bars.*

events. The extent to which the stain may recognize reversible as well as irreversible alterations also is not known. Longitudinal experiments that include estimates of the total number of neurons in specific brain regions will be important in addressing such questions.

Third, we have not measured the intensity of staining; rather, we have relied on estimates of the volume of intense staining. Consequently, there remains a subjective component to the volume determination. However, the authors' impression is that intensity and volume are highly correlated and the subjective component has only a minor, if any, influence on estimates of the volume of degeneration. We are currently developing an automated process for objectively determining the intensity and volume of staining.

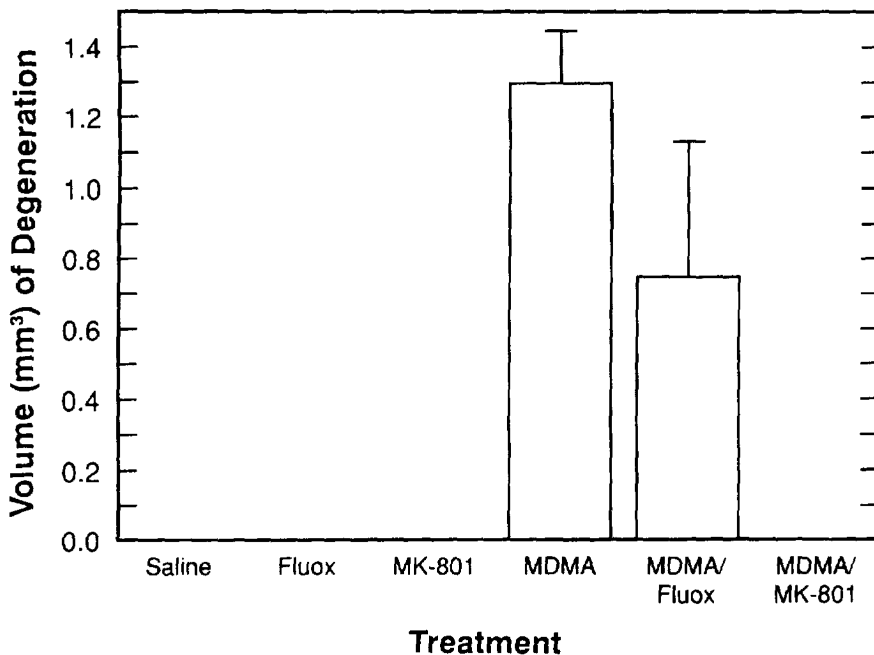

FIGURE 7. *Effect of fluoxetine (fluox) (5 mg/kg) and MK-801 (1 mg/kg) given 30 minutes prior to each of four doses of MDMA (100 mg/kg) on MDMA-induced silver staining. All groups included four animals. Animals received either saline or MDMA and were pretreated with either MK-801 or fluoxetine 30 minutes prior to MDMA. All animals were sacrificed 48 hours after the last dose. Note that fluoxetine pretreatment partially blocked MDMA-induced silver staining; MK-801 eliminated MDMA-induced silver staining. Standard errors indicated by vertical bars.*

Finally, this analysis was not exhaustive because the survey was restricted to selected sections through a segment of the cerebrum in which unambiguous staining was present. More extensive studies addressing the extent and nature of staining in a more precise manner are needed to fully characterize MDMA-induced silver staining.

CONCLUSIONS

Nonetheless, our current results show that maps of silver staining can demonstrate dose-response relationships. These maps of silver staining render a profile of MDMA-induced damage that differs from that engendered by evidence from neurochemical and immunohistochemical studies with respect

to two important features. First, MDMA-induced injury as revealed by silver staining is not restricted to serotonergic neurons. Second, the spatial pattern of injury as revealed by silver staining does not correspond to the spatial pattern of MDMA-induced alteration in serotonergic innervation. These two features of MDMA-induced injury highlight the potential involvement of nonaminergic mechanisms in MDMA neurotoxicity.

MDMA-Induced injury as Revealed by Silver Staining Is Not Restricted to Serotonerglc Neurons

Consistent with a previous report (Commins et al. 1987*a*), we have observed that MDMA induces silver staining of cell bodies, axons, and terminals in the neocortex. Because immunohistochemical studies have not revealed serotonergic perikarya within the neocortex (Lidov et al. 1980; Steinbush 1981) the staining of perikarya constitutes evidence of MDMA-induced injury to nonserotonergic neurons.

Additional evidence that nonserotonergic processes are also injured by MDMA comes from the observation that fluoxetine blocked part, but not all, of the MDMA-induced staining. Fluoxetine pretreatment reduces the volume of intensely stained tissue by approximately half and also reduces the intensity of staining throughout the stained region. Staining of terminals, axons, and cell bodies was still observed after fluoxetine pretreatment. Although we have not established a dose-response relationship for fluoxetine blockage of MDMA-induced silver staining, the dose of fluoxetine used, 5 mg/kg, was sufficient to prevent the serotonin depletion produced by the dose, 100 mg/kg of MDMA (J.P. O'Callaghan, D.B. Miller, and K.F. Jensen, unpublished observations). These observations support the conclusion that a substantial proportion of the degenerating terminals, axons, and cell bodies are nonserotonergic.

The Spatial Pattern of MDMA-Induced Injury as Revealed by Silver Staining Does Not Correspond to the Spatlal Pattern of Alterations In Serotonin Innervation

The regional pattern of reduction in serotonin is consistent with the idea that MDMA's effect on serotonergic neurons is selective for their axons and terminals (Wilson et al. 1989; Mamounas and Molliver 1988; O'Hearn et al. 1988; Ricaurte et al. 1988; Molliver et al. 1990). The MDMA-induced loss of uptake binding sites is largely in agreement with this idea (De Souza et al. 1990; Battaglia et al. 1987, 1991; Battaglia and De Souza 1989; De Souza and Battaglia 1989; Insel et al. 1989). Molliver and coworkers (Wilson et al. 1989; O'Hearn and Molliver 1984; Mamounas et al. 1991) have proposed that MDMA is even more selective, damaging primarily a subpopulation of serotonergic axons. They report that fine serotonergic axons originating in the dorsal raphe are preferentially vulnerable to MDMA, whereas coarse serotonergic axons from median raphe are resistant to

MDMA. Differences in the pattern of innervation between the fine and coarse fibers are most striking in the olfactory bulb, hippocampus, and entorhinal cortex where immunohistochemical studies have demonstrated a preferential loss of staining of these fine fibers following para-chloroamphetamine (PCA) or methylenedioxyamphetamine (MDA) (Mamounas et al. 1991).

The regional distribution of MDMA-induced silver staining does not correspond to the regional distribution of the vulnerable fine fibers. MDMA-induced silver staining is limited primarily to the frontoparietal cortex. It sometimes involves regions of posterior neocortex and striatum and is rarely observed in thalamus. Because terminal-like staining occurs frequently in the olfactory bulb and sometimes in the hippocampus of control animals, the possibility of MDMA-induced injury in these regions cannot be excluded. However, the alterations in serotonergic innervation revealed by neurochemical and immunohistochemical studies are far more widespread than the restricted damage to the frontoparietal cortex revealed by silver staining.

Within the neocortex, the vulnerable fine fibers can be seen throughout the cortical lamina but are particularly dense in layer IV (Blue et al. 1988; Kosofsky and Molliver 1987). MDMA, MDA, and PCA appear to selectively eliminate the staining of these vulnerable fine fibers (Steinbush 1981; O'Hearn and Molliver 1984; Mamounas et al. 1991). In contrast, the MDMA-induced silver staining is most pronounced in the upper layers, whereas at higher doses it becomes robust in layers III to V. Thus, in contrast to its more restricted regional pattern, silver staining appears to have a more extensive involvement within the frontoparietal cortex than alterations in the immunohistochemical labeling of serotonergic axons. Consistent with these findings, corresponding differences between neurochemical markers of dopamine and silver staining have been described for d-amphetamine (Ryan et al. 1990).

There are several interpretations that could account for the differences in the patterns of alterations revealed by serotonin immunohistochemistry and silver staining. One is that a reduction in the density of immunohistochemically labeled serotonergic processes can in part be accounted for by a loss of transmitter from intact axons, rather than solely by degeneration. A similar argument has been proposed for p-chloro-N-methylamphetamine (Lorez et al. 1976). Another consideration that may account partially for the results obtained with the two methods is that silver staining reveals damage to both serotonergic and nonserotonergic neurons. The contribution of nonserotonergic elements would not be detected by assays selective for assessing serotonin innervation.

There are other interpretations of these differences. One is that fine serotonergic degenerating axons do not take up silver stains. This assertion, although possible, has not been substantiated. However, it does highlight technical limitations of the anatomical methods used to assess MDMA-induced neural

degeneration. There are important limitations to both silver stains and immunohistochemistry. Silver stains do not distinguish between serotonergic and nonserotonergic degenerating axons. Serotonin immunohistochemistry cannot label intact axons that have lost their serotonin as a result of the pharmacological treatments. Thus, neither silver stains nor immunohistochemistry can by themselves discriminate the relative vulnerability of serotonergic and nonserotonergic elements to MDMA. Estimating the relative involvement of different populations of neurons awaits the development of techniques that can assess neural damage comprehensively while simultaneously providing the neurochemical identity of degenerating elements.

Although the precise identity and full complement of neurons vulnerable to MDMA require further characterization, several hypotheses have been put forward to account for the neurotoxicity of MDMA as well as other substituted amphetamines (for reviews, see Fuller 1989; Stone et al. 1991). The majority of these hypotheses are based on the idea that amphetamines are toxic to particular neurochemically defined classes of neurons, possibly through the formation of neurotoxins such as 6-hydroxydopamine and 5,6-dihydroxytryptamine (Commins et al. 1987*b*, 1987*c*; Axt et al. 1990). Our current observation that MK-801, an N-methyl-D-asparate (NMDA) receptor antagonist, blocks MDMA-induced silver staining suggests that there may be several alternate, nonserotonin-specific mechanisms by which substituted amphetamines can cause neural injury. The most obvious possibility is through an excitotoxic mechanism (Sonsalla et al. 1989; Olney et al. 1987). Interestingly, Carlsson has proposed that the neurotoxicity of the amphetamines may result from "uncontrolled positive feedback" from the striatum to the cortex through the thalamus. Such feedback could involve dopamine (cortical projections to the nigra or as presynaptic terminations on dopaminergic nigrostriatal afferents) or serotonin (cortical projections to raphe or as presynaptic terminations on serotonergic afferents to striatum) with the corticofugal projections being glutaminergic. Thus, one possible action of MK-801 could be to restabilize positive feedback, preventing a cascade of overstimulation. This hypothesis is consistent with the observation that MK-801 attenuates amphetamine-induced dopamine release (Weihmuller et al. 1991). It is also consistent with the currently observed incidence of silver staining in cortex, striatum, and thalamus following MDMA administration.

Degeneration blocked by MK-801 could also involve mechanisms other than excitotoxicity. MDMA-induced alteration in thermoregulation may be involved (Miller 1992; Gordon and Rezvani, in press). MK-801 can induce hypothermia that can protect against insults such as hyperthermia (Buchan and Pulsinelli 1990). Interestingly, MDMA-induced hyperthermia is also blocked by 5-HT2 receptor antagonists, and at high doses this effect can be differentiated from long-term reductions in serotonin (Schmidt et al. 1990).

Hyperthermia is not the only peripheral influence that could alter the extent of damage to the brain. Cerebral blood flow is modulated by serotonin (Gilman et al. 1990) and influenced by MK-801 (Roussel et al. 1992). Stress can alter brain tryptophan hydroxylase activity (Singh et al. 1990*a*, 19906; Boadle-Biber et al. 1989) and neocortical dopamine metabolism (Claustre et al. 1986). Because dopamine is involved in the action of substituted amphetamines on serotonin innervation (Stone et al. 1988; Schmidt et al. 1985, 1991), stress may play a role in the observed cortical damage. Indeed, individual variability in stress response (Miller 1992), particularly to multiple toxicant injections, also may play a role in the variability reported for several parameters altered by substituted amphetamines (Seiden 1991; Axt and Molliver 1991; Ryan et al. 1990; O'Dell et al. 1991), including that of silver staining in the striatum observed in the present study.

Finally, it is likely that several of MDMA's actions act in concert to produce silver staining as well as alterations in serotonergic innervation. Hypotheses that focus solely on the unique vulnerability of a particular neurochemically defined class of neurons are not likely to reveal a *more* complete understanding of such a complex phenomenon. Conversely, neural degeneration may not be the only means by which substituted amphetamines produce long-term alterations in neurotransmitter levels in the brain.

In summary, we have made significant steps toward quantifying a historically subjective phenomenon, the staining of degenerating neurons with silver. We have shown how maps of silver staining can be valuable in assessing the dose-dependent nature of toxicant-induced brain damage. With refinement, such maps can help identify components of the brain's complex circuitry that are vulnerable to damage by exposure to toxicants.

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DISCUSSION

Q (Molliver): Are you suggesting that this degeneration after MDMA is due to a neurotoxic effect of the drug?

A: Yes.

Q (Molliver): I feel uncomfortable with that. What is the mortality of your animals, given the huge doses you are giving?

A: We get very little mortality with the MDMA.

Q (Molliver): With dosages that high, you can get hyperthermia, hypertension, ischemia, and breakdown of the blood-brain barrier, in addition to pulmonary hypertension with hypoxia. At doses that high, one wonders whether you are seeing a specific drug effect. It is like the effects of cocaine, d-amphetamine, and methamphetamine, where people found vasculitis and ischemia, etc., with such high doses.

A: Certainly the fact that the doses are so high brings in the possibility about the specificity of the drug, in the sense of how it is being mediated-what kinds of things are kicking in at that kind of dose?

Q (Molliver): That is the dose range that is used to model experimental hypertension.

A: Right. But the time course, I think, what we are seeing is unlikely to be due to those kinds of secondary effects. The fact that we can see it after two doses? So it is within 12 to 16 hours.

Q (Molliver): You can get cerebral infarcts after one dose.

A: Right, but these rats are clearly healthy, by all criteria.

Q (Tilson): Wouldn't you expect the pattern to be more diffuse if that was the case?

A (Molliver): Not necessarily.

Q (Jensen): Aren't things like the hippocampus more susceptible to those kinds of infarcts?

A (Molliver): That's true; the hippocampus is more susceptible to ischemia.

Q (Tilson): How consistent are the changes you see in a population of rats? The slides that you have shown-are those representative of populations of rats?

COMMENT (Landfield): All slides are typical examples. (Laughter)

A: There is a certain degree of variability within a given dose group or in a given survival time. The degree of overlap between different doses is minimal, at least within the ranges that we've used. So that we can distinguish different dose levels, I would say, fairly consistently.

Q (Tilson): I was thinking about a pattern within the dose group, where there is always that area of the cortex, always. . .

A: Yes. Absolutely. In fact, the degree of damage that changes from dose is an increase in size of the area. The area itself never changes.

Q (Molliver): Did you look at the levels of neurotransmitters in the cortex, for example, serotonin?

A: Yes, we did.

COMMENT (Miller): Also, the hyperthermia and heat retention aren't as high when rats are in wire bottom cages,

Q (Molliver): I know, but did you measure temperature?

A (Miller): We have. If they are in plastic cages with wood chip bedding, they go up to 42° core temperature. If they are in wire bottom cages, they usually don't go any higher than 38°.

Q (Molliver): The main effect that I am concerned about is the sympathetic response due to peripheral release of catecholamines. Rats are sensitive to pulmonary hypertension, and the pulmonary vasculature can vasoconstrict.

Q (Jensen): Can they be protected?

A (Molliver): Possibly by an extensive sympathectomy and adrenalectomy. I think that Jim Gibb did that study years ago, and he found that adrenalectomy protected animals against MDA toxicity.

A (Gibb): If you give reserpine beforehand, you don't get the effect because you don't have anything to cause the effect.

COMMENT (Seiden): On the other hand, we've potentiated with reserpine.

COMMENT (Gibb): I am not sure when you gave yours. Did you give yours before? How long before?

COMMENT (Seiden): About 24 hours.

COMMENT (Ricaurte): I think the experiment has been done with AMT [alphamethyl tyrosine], where animals pretreated with AMT and then given a high dose of methamphetamine will not show silver degeneration in the striatum or the somatosensory cortex. In support of your idea, I would just like to reinforce that, because there is no question that the silver methods have a great potential for detecting degeneration. As you have emphasized, a major question is how much of that degeneration is specific in nature so that you could relate it to the catecholamine and monoamine systems and how much is related to nonspecific effects (ischemia, seizures, hyperthermia)'? My suspicion is that some of what has been shown in the cortex is in fact "nonspecific." It may be an interesting phenomenon in and of itself in terms of how it is mediated. But it is not the kind of serotonergic or dopaminergic neurotoxicity that is produced at lower doses. In the approaches that we use, one was with AMT and the other was combining the silver method with the pharmacological approach whereby we would pretreat animals with a dopamine reuptake blocker which we knew protected against the dopamine depletion induced by methamphetamine. We could show that such treatment also prevented the appearance of the silver degeneration in the striatum. My point is that the onus is to really demonstrate the specificity.

A (Jensen): It is true. Every drug does, and the question is what is the specificity and how is it. I should mention that fluoxetine will partially block the degeneration. Significant portions of degeneration are blocked by fluoxetine.

COMMENT (Seiden): I would like to take issue about every drug. With cocaine, in spite of approaching the doses that the animals can barely survive, we don't see neurotoxicity.

COMMENT (Gibb): Lew, that causes hyperthermia, right?

COMMENT (Molliver): It is very transient and is mild.

COMMENT (Gibb): Yes, but those doses that he is talking about cause hyperthermia.

COMMENT (Molliver): I think Karl clearly sees degeneration. I think this is of considerable interest. Perhaps it is even more of interest in terms of the clinical

aspects of drug abuse-induced toxicity seen in the emergency rooms, where the most common situation is vascular-mediated toxicity.

COMMENT (Landfield): As you yourself pointed out, these are new techniques, and you have to determine whether these are temporary pathologies or permanent. I would suggest possibly counting cells in some of these tissues. We all need to begin to quantify just how much degeneration this staining actually reflects.

COMMENT (Jensen): In fact, we are talking about doing an experiment with MPTP [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine], using standard morphometric methods to count the number of cells in pars compacta and seeing if that doesn't account for a loss in corresponsive cells that we see stained with cupric silver in the early time period. If there were major vascular events at these high doses, I would expect, based on ischemia and stroke models, to see pretty major cerebral gliosis. If you compromise the blood-brain barrier in a fairly major way and let in a lot of lymphokines and astrocyte mitogens, it would leave a fairly broad glial scar; we don't see that, and I will show you that with astrocytes. I think that one other thing that may be of equal consideration is what Dr. Heimer alluded to with some of his examples. When you have this extended damage, you almost have to be concerned about transsynaptic effects as well. The proximate site of the toxicant may be one set of cells, which upon degeneration will result in the damage of other cells. So you may end up with a cascade, particularly with these high doses, that may be dependent upon the presence of compound, but not directly related to the toxicant.

Q (Molliver): Did you see any intercerebral hemorrhages?

A (Jensen): No, we perfuse.

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Reactions of 5-HT Neurons to Drugs of Abuse: Neurotoxicity and Plasticity

Mary Ann Wilson, Laura A. Mamounas, Kenneth H. Fasman, Karen J. Axt, and Mark E. Molliver

INTRODUCTION

Many drugs of abuse that have mood-altering, mood-elevating, or hallucinogenic effects have direct pharmacologic actions on serotonergic (5-HT) neurons, particularly at 5-HT synaptic terminals (Aghajanian et al. 1970; McCall and Aghajanian 1980; Trulson et al. 1981; Jacobs 1984; Glennon et al. 1984; Rasmussen and Aghajanian 1986; Fuller 1986; Campbell et al. 1987). Several of the psychotropic amphetamine derivatives, such as p-chloroamphetamine (PCA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), and fenfluramine, have been shown to release 5-HT from serotonergic axon terminals (Gallager and Sanders-Bush 1973; Sanders-Bush and Martin 1982; Fattaccini et al. 1991; Johnson et al. 1991) and can also cause 5-HT axon terminals to degenerate (Battaglia et al. 1987; O'Hearn et al. 1988; Mamounas and Molliver 1988; Molliver and Molliver 1990). However, unlike 5,7-dihydroxytryptamine (5,7-DHT), which is neurotoxic to all types of serotonergic axons as well as serotonergic cell bodies, the amphetamine derivatives have selective neurotoxic effects that are restricted to a particular subset of 5-HT axons. Furthermore, the vulnerable and resistant axons can be distinguished on the basis of anatomic characteristics (O'Hearn et al. 1988; Wilson et al. 1989; Mamounas et al. 1991).

ANATOMIC FEATURES OF SEROTONERGIC PROJECTIONS

The serotonergic innervation of the forebrain arises from neurons in the raphe nuclei, located in the mesencephalon and pons (Dahlstrom and Fuxe 1964; Conrad et al. 1974; Bobillier et al. 1975; Moore and Halaris 1975; Moore et al. 1978; Azmitia and Segal 1978). Serotonergic axon terminals are found throughout the forebrain; however, there are regional and laminar variations in the distribution of 5-HT axons (Lidov et al. 1980; Morrison et al. 1982; Takeuchi and Sano 1983; Takeuchi et al. 1983; Kosofsky et al. 1984). The morphology of serotonergic axons has been examined in multiple species, and several morphologic axon classes have been identified (Chan-Palay 1977; Köhler et al. 1980; Lidov et al. 1980; Sano et al. 1982; Zhou and Azmitia 1986; Kosofsky and Molliver 1987; McLean

and Shipley 1987; Mulligan and Török 1988). Thick, nonvaricose axons, which are commonly found in white matter, are considered to be preterminal fibers. The other two types of 5-HT axons are varicose, but they differ in caliber and in the size of varicosities. *fine axons* with extremely small granular or fusiform varicosities are the most abundant 5-HT axon type in most areas of forebrain. *Beaded axons,* which have large, round varicosities and very fine intervaricose segments, are found in more restricted locations. Kosofsky and Molliver (1987), utilizing anterograde transport methods, have demonstrated that fine 5-HT axon terminals arise from the dorsal raphe nucleus and beaded 5-HT axon terminals arise from the median raphe nucleus. That study provided the first evidence that the different classes of axon terminals belong to distinct projection systems, with origins in different nuclei. The distribution of fine and beaded axon terminals has recently been studied in detail in rats (Mamounas et al. 1991) and in primates (Wilson et al. 1989; Hornung et al. 1990; Wilson and Molliver 1991). In most areas of the forebrain, fine 5-HT axons form a dense terminal plexus with subtle variations in density among different regions and cortical layers. However, beaded axons are markedly restricted in distribution, occurring predominantly in the outer neocortical laminae and in limited zones within particular structures such as the hippocampus, amygdala, entorhinal cortex, and olfactory bulb (figure 1). Beaded axons also form an extremely dense plexus lining the ependymal surface of the cerebral ventricles in both species. In several species, beaded axons have been observed to form pericellular arrays, or “baskets” (Mulligan and Török 1987, 1988; Hornung et al. 1990) intimately surrounding GABA-ergic neurons in some locations within the cortex (Köhler 1982; Török et al. 1988; Török and Hornung 1988; Freund et al. 1990; DeFelipe et al. 1991). Although no other specific postsynaptic targets of fine or beaded axons have yet been identified, the distinctive distributions of fine and beaded 5-HT axons suggest that these two axon classes influence different postsynaptic targets in the forebrain. For example, the markedly different laminar distributions of these two types of 5-HT axons within the cerebral cortex suggest that fine and beaded 5-HT axons are likely to terminate on different elements of cortical circuitry. In addition, these 5-HT projections can be differentiated pharmacologically: O’Hearn and colleagues (1988) have shown that fine and beaded 5-HT axons are differentially vulnerable to the neurotoxic effects of amphetamine derivatives such as MDA. These studies indicate that there are two parallel systems of serotonergic projections, differing in axon morphology, origin, distribution, and vulnerability to selective neurotoxins; they are likely to have different postsynaptic targets and subserve different functions.

AMPHETAMINES AND AXON LOSS

The authors have attempted to determine what methods may be useful for detecting and characterizing the neurotoxic effects of psychoactive compounds such as amphetamine derivatives to develop and validate criteria of neurotoxicity that are applicable to multiple neuronal systems, It is important to evaluate the

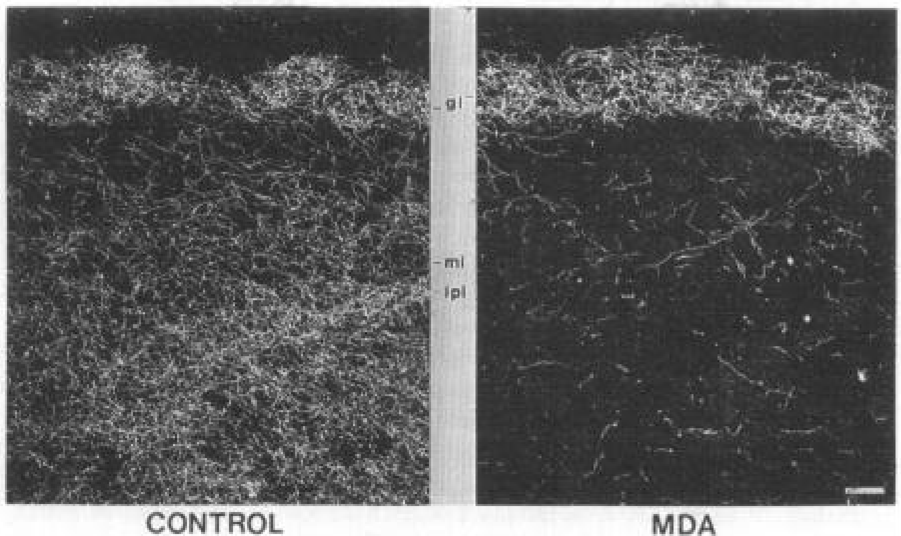

FIGURE 1. *Fine and beaded 5-HT axons in the olfactory bulb are differentially vulnerable to neurotoxic amphetamines. In control animals (left panel), fine and beaded 5-HT axons are segregated within different layers of the olfactory bulb: Fine axons ramify extensively within the infraglomerular layers; beaded axons are localized predominantly in the glomeruli where they form dense, convoluted arrays. Neurotoxic amphetamines such as PCA or MDA cause a marked loss of fine 5-HT axons, while sparing beaded axons (right panel). After drug treatment, beaded 5-HT axons in the glomerular layer remain intact, whereas fine axons degenerate, leaving the infraglomerular layers nearly devoid of serotonergic axons.*

KEY: gl=glomerular layer; mi=mitral cell layer; ipl=internal plexiform layer; 5-HT immunocytochemistry, darkfield photomicrograph, bar=100 µm

SOURCE: Mamounas, L.A.; Mullen, C.A.; O'Hearn, E.; and Molliver, M.E. Dual serotonergic projections to forebrain in the rat: Morphologically distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. *J Comp Neurol* 314:558-586, 1991. Copyright © 1991 by Wiley-Liss. Reprinted by permission of Wiley-Liss, a division of John Wiley and Sons, Inc.

spectrum of neuronal and nonneuronal effects produced by these drugs in terms of biochemical, pharmacologic, and morphologic criteria. These parameters include neurotransmitter content and synthesis, the density and activity of membrane proteins such as neurotransmitter uptake carriers, the

integrity of cytoskeletal elements, the morphologic characteristics of neuronal cell bodies and processes, and the status of nonneuronal cells such as microglia and astrocytes that may become activated or proliferate in response to neuronal damage. It is also important to carefully examine the time course of the effects of a particular drug and of the neuronal and nonneuronal responses to damage. Drug-induced changes in pharmacologic parameters such as neurotransmitter levels, synthesis, or uptake may be either transient effects, from which neurons can readily recover, or persistent changes that reflect damage to neuronal structure. In addition, certain phases of the neuronal response to a neurotoxin, such as the period of active degeneration of axons, may occur over a limited period; other effects, such as the induction of certain microglial markers, may be delayed and may vary depending on the nature of the damage that has occurred.

The response of fine serotonergic axons to PCA and related amphetamine derivatives exhibits several temporal phases marked by particular functional changes. Initially, a severe depletion of 5-HT content in forebrain occurs; after a single, moderate drug dose, 5-HT content may recover to near-normal levels (Sanders-Bush et al. 1975; Schmidt 1987). This acute recovery is transient, and the ensuing loss of 5-HT content and other serotonergic markers persists for weeks or months Although the initial reduction in 5-HT levels is thought to result from release of 5-HT and inhibition of reuptake, degeneration of 5-HT axons has been proposed as a likely explanation for the persistent decreases in 5-HT levels observed after treatment with PCA or fenfluramine (Neckers et al. 1976*a*, 1976*b*; Harvey et al. 1975; Clineschmidt et al. 1978). However, others have postulated that long-lasting deficits could be explained as depletion of neurotransmitter caused by 5-HT release combined with inhibition of uptake and synthesis (Sarkissian et al. 1990).

In a series of recent studies, both biochemical and anatomic results in animals treated with several related amphetamine derivatives have provided evidence in support of axonal degeneration. After animals are treated with PCA, MDA, MDMA, or fenfluramine, there are reductions in a number of serotonergic parameters that persist for weeks to months after treatment. These changes include large, persistent reductions in:

- Levels of 5-HT and its metabolite, 5-hydroxyindole-acetic acid (5-HIAA) (Sanders-Bush et al. 1975; Neckers et al. 1976*b*; Clineschmidt et al. 1976; Kleven and Seiden 1989)

- The activity of the 5-HT synthetic enzyme, tryptophan hydroxylase (Sanders-Bush et al. 1975; Neckers et al. 1976*b* Stone et al. 1986; Schmidt and Taylor 1987)

- The activity and density of 5-HT uptake sites, as determined in synaptosomal or autoradiographic preparations (Sanders-Bush et al. 1975; Battaglia et al. 1987)

- The density of 5-HT immunoreactive axons (O'Hearn et al. 1988; Mamounas and Molliver 1988; Wilson et al. 1989; Appel et al. 1989; Molliver and Molliver 1990; Mamounas et al. 1991)

- Retrograde axonal transport, demonstrated by labeling of raphe neurons with a retrogradely transported fluorescent tracer (Fluoro-Gold [FG]) (figure 2) (Mamounas and Molliver 1988; Fritschy et al. 1988; Molliver and Mamounas 1991; Axt and Molliver 1991)

These studies identify markers of axon viability that are lost after drug treatment: neurotransmitter content and synthetic machinery, the mechanism for reuptake of released neurotransmitter, and the cytoskeletal elements required for transport of compounds from the axon terminal back to the parent cell body. In addition, positive evidence of axonal degeneration is provided by morphologic studies (figure 3), which reveal numerous extremely swollen, abnormally shaped, and fragmented axon terminals at short survivals (1 to 3 days) after drug treatment (O'Hearn et al. 1988; Wilson et al. 1989; Molliver and Molliver 1990; Axt et al. 1990, 1992). These abnormal morphologic features are characteristic of degenerating axons. The time when degenerating axons are most numerous corresponds with the onset of the long-lasting phase of serotonin loss noted above; this transient phase of degeneration produces a persistent denervation. Taken together, this constellation of abnormal findings observed after treatment with amphetamine derivatives indicates that 5-HT axon terminals are damaged by these compounds and subsequently degenerate.

The mechanism of the neurotoxic effects of amphetamine derivatives is as yet unidentified; however, agents such as citalopram and fluoxetine, which selectively inhibit 5-HT uptake, also block the degeneration of 5-HT axon terminals after amphetamine treatment (Fuller et al. 1975; Schmidt and Gibb 1985). It has not been determined whether these amphetamine derivatives have direct neurotoxic effects or whether a metabolite of the drug (or of released monoamines) is responsible for the neurotoxicity observed in vivo. Mediation by a toxic metabolite is suggested by in vitro studies (Molliver et al. 1988) and by the lack of neurotoxicity observed after direct intracerebral injection of MDA, MDMA (Molliver et al. 1986), or PCA (Berger et al. 1990). In addition, prior depletion of both peripheral and central stores of 5-HT with reserpine and the 5-HT synthesis inhibitor parachlorophenylalanine blocks the neurotoxic effect of PCA (Berger et al. 1989, 1992). These studies suggest that a toxic metabolite of 5-HT or of a catecholamine (Schmidt et al. 1985; Commins et al. 1987; Stone et al. 1988) may be formed after treatment with PCA and related compounds.

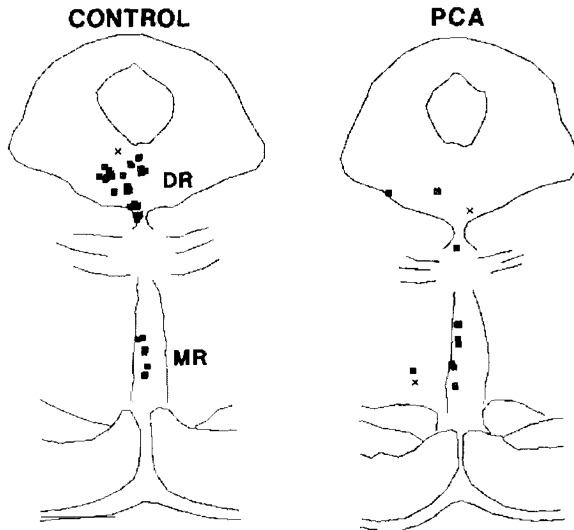

FIGURE 2. *Dorsal and median raphe projections to the olfactory bulb are differentially vulnerable to neurotoxic amphetamines. Retrograde axonal transport of the fluorescent dye FG was utilized to identfy raphe neurons that project to the o/factory bulb. After large injections of FG in both the glomerular and infraglomerular layers of the olfactory bulb, retrogradely labeled raphe neurons were mapped in control and PCA-treated rats (10 mg/kgx2; administered 2 weeks prior to FG injections). In control rats, many FG-labeled neurons (depicted by an X) are found in both the dorsal and median raphe nuclei; most of these neurons are serotonergic (an X surrounded by a box). Serotonergic axons that are ablated by PCA do not transport the tracer to their cell bodies; thus, changes in the distribution of FG-labeled cells in the raphe nuclei after PCA treatment may be used to determine the brain stem origins of damaged vs. spared axons. Administration of PCA 2 weeks prior to FG injection reduces the number of retrogradely labeled 5-HT neurons in the dorsal raphe (77-percent reduction); the number of labeled neurons in the median raphe remains unchanged. These results indicate that the fine 5-HT axons in the olfactory bulb, which are selectively damaged by amphetamine neurotoxins such as PCA or MDA (see figure 1), arise from the dorsal raphe nucleus, whereas the beaded 5-HT axons, which are preferentially spared, arise from the median raphe nucleus.*

SOURCE: Molliver and Mamounas 1991

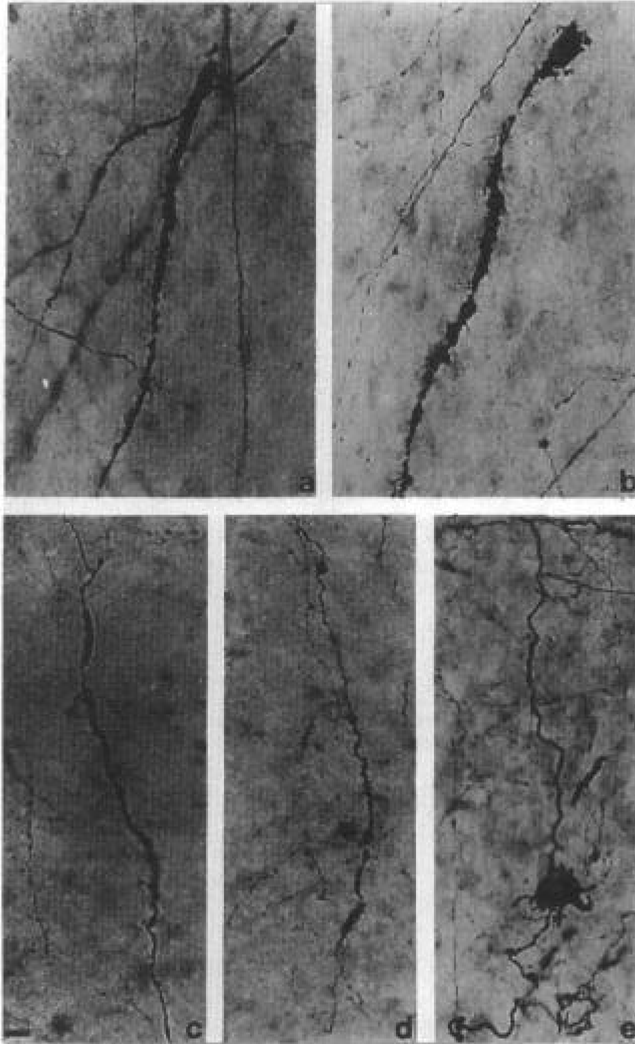

FIGURE 3. *Abnormal serotonergic axons in the forebrain of squirrel monkeys 2 days after dexfenfluramine administration. Dexfenfluramine (5 mg/kg, subcutaneous) was administered to squirrel monkeys twice daily for 4 days. Two days later serotonin immunocytochemistty revealed numerous 5-HT axons with abnormal morphologic features that are characteristic of degenerating axons. Panels A and B: engorged preterminal axon stumps. Panels C through E: extremely swollen, abnormally shaped, and fragmented axon terminals. Brightfield photomicrographs, bar= 10 µm.*

PCA has been shown to inhibit monoamine oxidase (Fuller 1966); this effect may favor the formation of an abnormal, toxic metabolite by blocking the usual mechanism for monoamine metabolism.

Immunocytochemical studies, in addition to providing positive evidence of axon degeneration, have revealed that fine and beaded 5-HT axons differ in vulnerability to the effects of amphetamine derivatives. These two axon types have different, partially overlapping distributions, and the local effect of amphetamines on the 5-HT content in a particular region depends to a great extent on the proportion of fine vs. beaded 5-HT axon terminals in that area. Regions that are innervated primarily by fine 5-HT axons such as dorsal neocortex, have proven especially vulnerable, whereas regions innervated predominantly by beaded 5-HT axons, such as the amygdala, are relatively resistant. Moreover, these studies suggest that compensatory changes within surviving processes can mask the cytotoxic damage to fine axons when assayed by means of biochemical markers such as neurotransmitter levels, Because the nuclei of origin and the postsynaptic targets of fine and beaded axons are different, it is likely that these two projections subserve different functions; the functional effects of a chemical lesion restricted to one subset of 5-HT axons probably differ from the effects of a neurotoxin such as 5,7-DHT, which causes both systems to degenerate. Thus, the morphologic information provided by immunocytochemical methods may be useful in interpreting both biochemical and behavioral data concerning the effects of these neurotoxic compounds. Retrograde axonal transport methods also have proved useful and highly sensitive in detecting neurotoxic drug effects on a set of axon terminals that constitutes only a small proportion of the 5-HT afferents to a particular region. For example, the dorsal raphe projection to the motor trigeminal nucleus contributes a small proportion of the fibers to the dense plexus of 5-HT axon terminals within the nucleus; their loss after PCA treatment is not readily apparent in immunocytochemical preparations. However, the high anatomic resolution and sensitivity provided by retrograde transport methods allowed Fritschy and colleagues (1988) to detect the loss of the dorsal raphe projection to the trigeminal motor nucleus. Thus, although anatomic data are more difficult to quantify than biochemical measures, the spatial resolution and morphologic information obtained with neuroanatomic methods can be helpful in detecting and characterizing the neurotoxic effects of psychoactive compounds.

MORPHOMETRIC ANALYSIS OF AXON DENSITY

A method for quantitative analysis of neuroanatomic data is particularly useful to provide high sensitivity for the detection of neurotoxic effects, obtain dose-response curves, permit statistical analysis of results, and correlate these results with other measures. To that end, the authors have undertaken the development of a group of morphometric procedures for the semiautomated measurement of axon density within terminal fields as seen in the light microscope. The goal of

this method is to quantify morphologic parameters of axon density, including features such as the number of axon segments in a defined field, the cumulative length of the observed axons, and ideally, a breakdown of these parameters by axon type. This approach will then be applied to the problem of quantifying anatomic changes in 5-HT axons following drug treatment or other experimental perturbation. The results will be presented in a future report.

Although cell counting (and “blob” counting in general) is a well-examined problem in quantitative microscopy with several workable solutions, there are no accepted automated methods for measuring the morphologic features of arborized axons. (Counting the number of axons in a cross-section through a central nervous system [CNS] tract or a peripheral nerve has been explored, but that problem is reduced to simple blob counting.) High magnification is needed to resolve individual axons, but that introduces the optical limitation of a narrow depth of field. Generalized line-counting techniques are inadequate for dealing with tortuous axons that, at high magnification, move in and out of the shallow focal plane.

The methods that we have been exploring for measuring axon density involve the orderly application of a series of standard digital image-processing techniques to digitized video images obtained directly from microscope slides (figure *4,* panel A). These algorithms have been modified for the specific requirements of this task. Several heuristics based *on* the expected characteristics of axons in the images are introduced at critical steps in the image-processing procedures. For example, in spite of the tortuous path taken by many cortical axons, they can be treated as locally linear objects. Thus, to follow an axon through an image, small gaps are closed by keeping track of the most recent tracing direction. Other processing steps take advantage of the fact that the light intensity in these images varies sharply in the immediate vicinity of an axon. Thus, the variance of intensity levels around an axon in the image is a more useful segmentation measure than the binary intensity thresholding that is commonly used in image analysis algorithms to separate foreground objects from the image background. Techniques such as these allow obvious background regions to be discarded automatically, permitting the computationally intensive tracing algorithms to be restricted to areas that have a high probability of containing axons (figure 4, panel B).

We are continuing to develop and refine these techniques for analysis of the anatomic changes induced by the drugs under study. These methods will provide quantitative information indicative of cumulative axon length as an index of axon density. The accuracy of the methods is being evaluated based on comparison with manually traced images obtained from control and treated tissue sections.

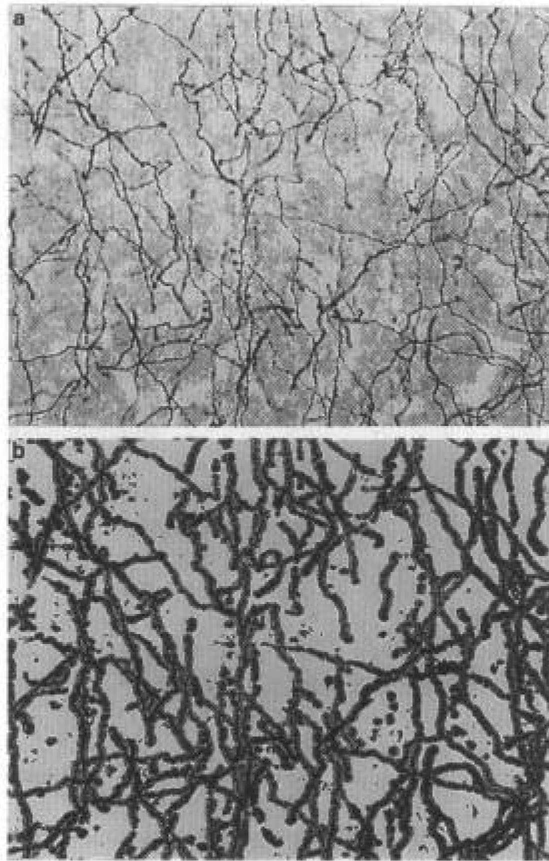

FIGURE 4. *Computerized image analysis of axon density: initial separation of axons from background. Axon density will be determined in video images obtained from light microscopic sections. This figure shows an initial step in processing a digitized image to separate stained axons from background. Panel A: Digitized image of serotonergic axons in parietal cortex of a control rat. Because the light intensity in these images varies sharply in the immediate vicinity of an axon, the variance of intensity levels around an axon in the image is a useful segmentation measure for separation of foreground objects from the image background. Panel B: An example of image segmentation based on local intensity variance: processed image with background regions removed. Removing large background regions from consideration greatly improves the performance of axon tracing algorithms. Tracing methods are considerably faster and more accurate when applied to the remaining regions.*

SPROUTING AND AGING OF SEROTONERGIC NEURONS AFTER NEUROTOXIC DAMAGE

As discussed above, certain psychotropic amphetamine derivatives, such as PCA and MDA, cause selective degeneration of one class of 5-HT axon terminals. Although fine 5-HT axon terminals degenerate, beaded 5-HT axon terminals are spared. In addition, preterminal 5-HT axons and 5-HT cell bodies are spared. The degeneration caused by MDA or PCA is followed by axonal sprouting, leading to slowly progressive reinnervation (Molliver et al. 1989; Molliver and Axt 1990; Mamounas and Molliver 1991). This gradual, partial reinnervation of forebrain proceeds over several months. The reinnervation of cerebral cortex progresses from rostral to caudal in a pattern that reiterates development: A bilaminar ingrowth of thick, straight axons in layers I and VI is followed by arborization of axon terminals within all layers of cortex. This gradual reappearance of fine 5-HT axon terminals and, in particular, the spatial pattern of reinnervation, provide additional, compelling evidence that the preceding loss of 5-HT axons was caused by a degenerative process and was not merely a depletion of neurotransmitter. The newly formed axon terminals have the morphologic features typical of fine 5-HT axons. If a second course of MDA is administered at this point, many of the regenerated axons are vulnerable to the neurotoxin (Wilson and Molliver 1990). Both the morphologic appearance of the regenerated axon terminals and their vulnerability to a second course of neurotoxin treatment provide support for the hypothesis that, after MDA-induced degeneration, there is regenerative sprouting of fine dorsal raphe axons rather than collateral sprouting of the spared, beaded median raphe axons.

Although amphetamine derivatives such as PCA and MDA damage 5-HT axon terminals, a loss of neuronal cell bodies has not been detected in the raphe nuclei. Furthermore, the monoaminergic neurons of the CNS exhibit an unusual capacity to respond to injury with a regenerative response; if the 5-HT cell bodies do not die, they can, through sprouting, give rise to new axonal projections. Because the spatial pattern of axonal ingrowth during regeneration reiterates that seen in perinatal development and the relative axon density observed in different forebrain areas is similar to that seen in untreated rats, it is plausible that there is partial recovery of functional serotonergic innervation. However, it has not yet been determined whether the new axon terminals form appropriate connections with their targets.

The process of reinnervation has been examined further in long-term studies of PCA-treated rats (Mamounas and Molliver 1991) where it exhibits several unexpected features. These studies indicate that, although fine 5-HT axons undergo regenerative sprouting during the first few months after treatment, the innervation density does not reach control levels in caudal areas of neocortex 6 months after drug treatment (Mamounas and Molliver 1991). Moreover, at longer survival times after PCA administration (6 to 12 months), the sprouting

appears to terminate, and there is a subsequent decrease in the density of fine 5-HT axons in cerebral cortex. Concomitant with this delayed decrease in axon density is the appearance of structurally abnormal 5-HT axons that are relatively thick and form highly tortuous endings. These aberrant tortuous 5-HT axons are not seen during the early period of recovery after PCA; however, 1 year after PCA administration, aberrant tortuous axons form a large proportion of the 5-HT axon plexus in cortex. Aberrant axons of this type are not seen in young control rats but appear with increasing frequency, thickness, and tortuousness in aged untreated rats (from 1 to 2 years old). Quantitative analysis indicates that, 1 year after PCA treatment, there is a twofold to threefold increase in the number of these aberrant tortuous 5-HT axons in neocortex when compared with age-matched control rats. Thus, despite the initial regenerative response of 5-HT neurons after PCA-induced injury, the normal 5-HT innervation pattern is never completely reestablished. Moreover, as noted above, this regenerative response is followed by a second phase of axon loss. This phenomenon of delayed axon loss, accompanied by the development of axon tangles, may represent accelerated aging of 5-HT projections. There are other possible causes for these late regressive changes, such as failure of regenerated axons to contact appropriate targets.

There are several different meanings implied by the term “neurotoxicity,” as used by various authors and investigators. In this discussion, the term refers to structural damage to nerve cells that results in degeneration within one or more compartments of a neuron, for example, the axon terminal. As described above, psychoactive amphetamine derivatives have multiple effects on 5-HT neurons, and it is important to distinguish between the acute pharmacological effects of these drugs and the irreversible degeneration of 5-HT axon terminals that may ensue. Acutely, these compounds evoke profound release of 5-HT combined with inhibition of neurotransmitter synthesis and uptake, all of which lead to depletion of the transmitter from axon terminals. These immediate effects on neurotransmitter levels appear to be reversible. Moreover, if the usual sequence of drug effects is interrupted by administration of an uptake inhibitor such as citalopram within a few hours of amphetamine administration, the affected neurons can be protected and rescued: Neurotransmitter levels return to normal, and there is no morphologic or biochemical evidence for a persisteni neurotoxic effect. However, in the absence of such pharmacologic intervention, the acute drug-induced depletion is followed by a relentlessly progressive degeneration of 5-HT axon terminals. During this second phase, swollen, fragmented nerve fibers provide positive morphologic evidence demonstrating that 5-HT axons degenerate after amphetamine treatment. The resulting denervation causes persistent decreases in multiple markers for viable 5-HT projections. These long-lasting consequences of amphetamine treatment reflect structural damage to the axonal compartment of a specific set of neurons and constitute a neurotoxic effect. Thus, the authors apply the term neurotoxicity to a drug effect that causes structural damage to neurons, whether it involves

degeneration of the cell body or is limited to degeneration of neuronal processes (e.g., axon, dendrites). This concept of neurotoxicity applies to any compound that produces neuronal degeneration, even though damaged processes may subsequently be replaced through compensatory sprouting.

RESPONSES OF NONNEURONAL CELLS TO NEURONAL DAMAGE

Astrocytic Response

Many studies have demonstrated that astrocytic scarring occurs after mechanical or severe degenerative damage in the CNS. In recent years, increases in levels of the intermediate filament protein glial fibrillary acidic protein (GFAP), a major cytoskeletal component in astrocytes, have been observed in association with reactive astrocytosis (Graeber and Kreutzberg 1986; Brock and O'Callaghan 1987; Tetzlaff et al. 1988; Gilmore et al. 1990; Zini et al. 1990; Wang et al. 1991; Zimmer et al. 1991). Biochemical assays of GFAP levels, measurement of GFAP mRNA levels, and immunocytochemical detection of GFAP have been used to measure astrocytosis (Brock and O'Callaghan 1987; Hajós et al. 1990; Tanaka et al. 1991; Kost-Mikucki and Oblinger 1991; Poirier et al. 1991); these measures have been proposed for use as an index of neurotoxicity (Brock and O'Callaghan 1987; Haglid et al. 1991; O'Callaghan and Miller, this volume). Marked increases in the size of astrocytes, the intensity of GFAP immunocytochemical staining, and the number of astrocytic processes have been observed after mechanical lesions and after neurotoxin exposure that produces substantial brain lesions. For example, the retrograde reaction seen in the facial nucleus after transection of the facial nerve (VII) evokes a substantial increase in GFAP synthesis within 1 week of the injury (Graeber and Kreutzberg 1986, 1988). O'Callaghan and colleagues (1990) have observed increases in GFAP levels after degeneration of the dense striatal dopamine plexus brought about by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Hence, there is good evidence to support an increase in GFAP levels associated with several types of neuronal injury. However, the astrocytic response may not be a sufficiently sensitive marker for detection of all types of neuronal damage or of less severe injuries such as the selective degeneration of sparsely distributed 5-HT axon terminals observed after PCA treatment.

In a series of experiments carried out in the authors' laboratory, the astrocytic reaction was evaluated using GFAP immunostaining in rats that had been treated with PCA (10 or 20 mg/kg, once per day for 2 days), at survival times ranging from 3 days to 3 weeks, Although PCA treatment produced extensive degenerative changes and a substantial loss of fine 5-HT axon terminals throughout the forebrain, no consistent alterations in GFAP immunoreactivity were observed with respect to the number of astrocytes, their staining intensity, size, or number of processes. These studies suggest that astrocytic responses visualized with GFAP staining may have limited sensitivity for the detection of

damage to sparsely distributed, fine axonal processes: moreover, a modest loss of fine axon terminals may not be a sufficient stimulus to evoke an astrocytic reaction, However, Frankfurt and colleagues (1991) have reported increases in both the number of GFAP-labeled astrocytes and GFAP levels after injections of the selective neurotoxin 5,7-DHT in the hypothalamus of adult rats. The latter lesion causes extensive degeneration of preterminal axons as well as axon terminals.

To further evaluate the usefulness of GFAP assays for detection of neurotoxic effects on monoaminergic projections, Dr. Karen Axt has recently carried out a series of studies in this laboratory, utilizing low-dose intraventricular injections of 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-DHT. These potent neurotoxins selectively ablate 5-HT axons, especially around the ventricles and in parietal and occipital cortex. In addition, both compounds produce a toxic effect on some dopamine projections in the striatum. The damage to dopamine terminals caused by 5,7-DHT can be prevented by pretreating the rats with nomifensine, a catecholamine uptake blocker. Thus, three types of lesions can be produced by injection of 5,6-DHT or 5,7-DHT: nonspecific mechanical damage along the path of the injection cannula, selective neurotoxic damage to 5-HT axon terminals, and damage to striatal dopamine axons. In animals treated with 5,6-DHT or 5,7-DHT alone, an increase in GFAP immunostaining was observed in the zone of mechanical damage along the cannula tract and in a region of the caudal striatum that exhibited a marked loss of dopamine axons. In other regions of forebrain that do not receive a prominent dopaminergic innervation (notably in parietal and occipital cortex), striking losses of 5-HT axons occurred without a notable increase in GFAP staining. Furthermore, when the neurotoxic effects of 5,7-DHT on dopaminergic axons were selectively blocked by pretreating with nomifensine, only minor changes in GFAP staining were found in the striatum apart from the region of mechanical damage produced by the injection cannula.

The finding that GFAP expression is not increased in regions with extensive 5-HT axon loss and is increased in the striatum primarily in response to a loss of dopaminergic axons suggests that the astrocytic reaction may not be detectable in all cases of neurotoxic effects on axons. Increases in GFAP staining are observed in regions of neuronal death, retrograde reaction of cell bodies, and degeneration of dense axonal projections. However, GFAP staining appears to have limited use in detecting damage to sparsely distributed, fine axonal projections such as the 5-HT projections to most areas of forebrain. Although an astrocytic reaction is often associated with neuronal death, the failure to detect astrocytosis or an increase in GFAP does not provide sufficient evidence to exclude neuronal injury or neurotoxicity.

Microglial Responses

Microglial cells are a heterogeneous class of cells in the CNS that respond to neuronal damage and have the capacity to proliferate and to become phagocytic. They were first detected in the brain with heavy metal stains by del Rio-Hortega (1932), but there has been controversy as to their origin and even whether they are normally resident in brain tissue. In the past several years there has been an increased interest in microglia, based on immunologic studies demonstrating that a number of antigenic markers expressed by peripheral macrophages and lymphocytes are found in brain cells with the same morphology as classic microglia, or "dendritic cells" (Perry and Gordon 1991). There is evidence that some phagocytic cells in the brain are derived from microglia that, in the past, may have been misidentified as astrocytes (Graeber et al. 1988*a*). Current evidence indicates that microglia are extremely numerous in the normal brain, are activated by neuronal injury, and may participate in the elimination of damaged neuronal processes and somata by phagocytosis.

Microglia exhibit several characteristic features that are altered in response to CNS injury. Activated microglia undergo morphologic changes and upregulation or novel expression of macrophage/lymphocyte antigens: under certain circumstances, they may proliferate or become phagocytic. The response to a particular injury varies, depending on the nature of the injury, for example, neuronal death vs. axotomy (Streit et al. 1989).

To assess the response of microglia to neuronal damage, it is necessary to develop reliable methods for identification of microglia in the resting state and after activation. A number of anatomic methods have been utilized to detect microglial activation. One of the most widely used is staining with either a specific lectin such as that derived from *Griffonia simplicifolia* seeds (Streit and Kreutzberg 1988) or with antibodies directed against monocytic markers. A monoclonal antibody directed against the type 3 complement receptor on the plasma membrane of macrophages, MRC OX-42 (Serotec), has been applied in several studies of microglial activation (Graeber et al. 1988*b*, 1989; Konno et al. 1989; Rinaman et al. 1991; Castellano et al. 1991; Sedgwick et al. 1991). Our initial findings with this antibody confirm that OX-42 is a highly sensitive marker for microglia in the resting and activated states. In rats treated with PCA, there is a notable increase in the OX-42 staining intensity of microglia and alterations in the morphology of microglial processes that are especially evident in hippocampus, striatum, and neocortex (Wilson and Molliver 1992).

To further characterize the responses of microglia to neurotoxic damage and identify indices of different states of glial activation following neuronal injury, the authors are evaluating the utility of other microglial markers. The following antisera have been selected for study: the Serotec mouse monoclonals OX-42,

described above; OX-1, directed against the rat leukocyte common antigen CD45; OX-6, directed against major histocompatibility complex (MHC) Class II antigen (la): OX-18, directed against an MHC Class I antigen; ED-l, directed against a monocyte/macrophage cytoplasmic antigen that may be a marker for lysosomal activation: and W3/25, directed against the rat homolog of the human CD4 antigen.

A well-characterized experimental system for the study of microglial activation, the facial nerve model (Graeber et al. 1988*b*), has been used to optimize staining parameters and examine the response of microglia to motor neuron axotomy. A retrograde cellular response (chromatolysis) is produced in the motor neurons of the facial nucleus, without damage to the blood-brain barrier, by transection of the facial nerve where it exits from the stylomastoid foramen. The motor neurons of the facial nucleus undergo a characteristic retrograde reaction indicative of degenerative changes and neuronal injury, but the cell bodies do not die; rather, they survive, and the transected axons may later regenerate.

The authors have examined the responses of microglial cells in the facial nucleus 4 to 8 weeks after facial nerve transection. Staining of the six microglial antigens noted above is increased in the facial nucleus on the side of the lesion when compared with the unlesioned side. In this experimental model, the antiserum revealing the largest differences vs. controls is OX-6, which is directed against the MHC Class II (la) antigen. Despite the lack of apparent cell death in this model, there is punctate staining on the lesioned side with the ED-1 antiserum, which may indicate lysosomal activation. The other antisera examined also appear to be useful markers for microglial activation, and we are evaluating all six markers for the detection of neurotoxic damage to 5-HT axon terminals. The nature and time course of microglial responses have been reported to vary after different kinds of lesions; therefore, it is important to utilize a wide variety of microglial markers and different survival times when attempting to characterize the microglial response to a particular class of injury. Preliminary results indicate that changes in the profile of microglial markers may be useful for detecting neuronal injury and may be more sensitive than measures of astrocytic activation for detecting selective damage to a small subset of the axons in the neuropil.

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DISCUSSION

Q: Have you seen a microglial reaction along the needle track?

A (Molliver): Yes, very striking.

Q (Landfield): Only the astrocytes have processes like those you showed.

A: Those are identical to the cells that del Rio Hortega identified as microglia, and they do not costain with GFAP, so they are not astrocytes.

Q (Landfield): The localization that you have shown in the hippocampus is very similar to the astrocyte localization.

A: In the hippocampus, astrocytes have a similar pattern

Q (Landfield): The microglia are truncated cells, and they are just not that frequent. There are many more astrocytes in the hippocampus, and the distribution you show looks much more like a stain for astrocytes. So I think there may be some cross-reactivity in your stain.

A: We were puzzled about that at first, because I did not believe that there were that many resident microglia in the brain. Now, there is much re-interest in microglia, and there are many recent papers. A number of laboratories, particularly G. Kreutzberg's group in Germany and several laboratories in England and the United States, have demonstrated abundant microglia with monoclonal antibodies directed against MHC markers.

COMMENT (Landfield): That is a problem with antibodies, I think, because at the EM [electron microscopy] level it is clear that is precisely what they are.

They have been well described: The microglia are dark cells, they are more rounded, and they don't have the long extensive processes you show. Also, If you don't see an astrocyte reaction, then you probably don't have degenerating axons. I would like to bring this up again in terms of what we were talking about before, about the need to have a secondary method to validate a decrease in staining; as Lew Seiden pointed out, a decrease in neurochemistry doesn't necessarily mean you have degeneration. In fact, if you did have such massive degeneration, I would be astounded if you didn't have loss of cells in the raphe itself. Why don't you have loss of cells? Why don't you have an astrocyte reaction? I would say that you may have a definite decrease in staining, and a decrease in neurochemistry, but it is still an open question whether you have degeneration. And I tend to doubt it from these data.

COMMENT (Molliver): That is an interesting point, and I am certainly open to discussion about this. Recently it has been found that there are two distinct populations of glial cells in the brain, and you can distinguish in the same section or in adjacent sections-those which stain with GFAP and those which stain with OX-42 or a battery of other monocyte markers for microglia. Recent studies from Kreutzberg and colleagues have shown that the microglia, rather than astrocytes, are the main glial cells that respond to degeneration.

COMMENT (Landfield): With semithin sections or EM, you can see all the cells that are there.

COMMENT (Olney): But you can't trace the whole process because they are so thin.

COMMENT (Landfield): No, you can't trace the whole process. But in many sections, the astrocytes may be very elongated, whereas you never or rarely see that in the dark cells: dark cell processes are just not that frequent. I can show you in any section of semithins, and you can clearly see where long processes are distributed. And the distribution shown here is like that for astrocytes.

COMMENT (Molliver): That is the point that I was trying to make. Based on the localization of distinct immune markers on microglia, we should consider a revision of the concept of these two cell types. For example, there is now a great deal of evidence that the AIDS virus selectively affects microglia and that it replicates in these cells. We see that microglia, not astrocytes, stain for these immune markers, common to macrophages.

As far as the degeneration process goes, several parameters need to be considered. I agree that the loss of staining by itself is not evidence for degeneration. However, consider, first, what the biochemical factors are.

There is a loss of transmitter, a loss of tryptophan hydroxylase activity, a loss of serotonin uptake. Those are independent factors. Uptake is a function of the plasma membrane; transmitter storage is a function of the synaptic vesicles; and synthesis is a function of the cytoplasmic protein, tryptophan hydroxylase. There is loss of axon transport, both anterograde and retrograde, indicating that cytoskeletal function is impaired. There appears to be no transport, storage, or uptake, and there is hardly any evidence for viability of 5-HT axons. Even that itself is not sufficient. These enormous, swollen, globular, fragmented axons that are 10 to 20 times larger than normal provide positive evidence for degeneration. One of the most striking results is seen in sagittal sections: 5-HT axons in the hypothalamus form large, bulbous, swollen endings, which have been described by Cajal after axotomy and by Bjorkland and Hokfelt after neurotoxic lesions of catecholamine axons. The combination of the loss of all evidence of viability, the presence of enormously swollen, fragmented axons, of growth cones with filopodia coming off, and the gradual pattern of sprouting over 8 to 12 months makes most comfortable with the interpretation that the 5-HT axon terminals have degenerated after treatment with PCA or MDA.

COMMENT (Landfield): There are more direct ways to study this. I am just saying this type of evidence reminds me a little bit of the early aging studies with Golgi silver stains. It was originally thought that the brains were massively degenerating in normal aging, but it has all turned out not to be true. In fact, you find growth of dendrites rather than the loss of dendrites in normal aging in some cases. Only in the very extreme cases of Alzheimer's disease have they seen clear attrition. So, many things that you see with staining are affected by tissue quality and stainability.

I am probably preaching to the converted, for most people here. Staining of fibers is interesting, but again, an important question is, do the raphe cell bodies stain? Do these lose staining? Because the answer may tell us whether the cells are degenerating.

COMMENT (Molliver): The raphe cell bodies seem to be normal, although we haven't yet obtained optimal morphology. George Ricaurte has reported evidence of inclusion bodies in the raphe cell bodies in the primate. We are continuing to look for other changes in these cells.

COMMENT (Landfield): If you could locate the cell bodies, you should be able, at the EM level or with other methods, to follow the axons.

COMMENT (Molliver): It is not technically feasible to follow axons over several regions in the EM in sections that are 700 angstroms. I am basing my interpretation of degeneration on the composite set of data such as loss of 5-HT uptake, loss of anterograde transport, loss of retrograde transport, and abnormal morphology.

COMMENT (Landfield): All those data also can be consistent with massive excitation-induced depletion of these cells and with fatigue or an exhaustive state.

COMMENT (Molliver): For several months?

COMMENT (Landfield): But on the other hand, it is very unusual to see this kind of massive axon degeneration and no change in the cell bodies and no astrocyte reaction.

COMMENT (Molliver): Damage to the trochlear nucleus is being studied by Hazel Murphy at the Medical College of Pennsylvania, as a model of the neuronal response to injury. They have found that the degree of injury is a function of the distance from the cell body that the axon is cut. If the axon is cut near the terminals, there is little reaction in the cell body. As you cut the axon closer to the cell body, you begin to see some changes. If you cut at the decussation of the trochlear nerve in the isthmus, then the cell bodies show degeneration. More distally they don't degenerate, but they reinnervate the superior oblique muscle.

COMMENT (Landfield): Did you study the loss of axon all the way back?

COMMENT (Molliver): The only axon loss that we detect is in the axon terminals. The preterminal axons in the MFB [medial forebrain bundle] are not only intact, but the axons are slightly thickened, as if there were damming of transported material.

Q (Jensen): Mark, given the amount of depletion that has taken place, how much information do you think there actually would be in histochemistry? In other words, if you're labelling the serotonin, that is gone. Right?

A: Only in terminals.

Q: Okay, only in terminals, but . . .

A: There are increased amounts in the preterminal axons.

Q: Right. But in the cortex, you get the depletion probably before you get degeneration. Right?

Q: Would you say they are concomitant?

A: No, there is a specific sequence of transmitter change that has been eloquently described by Chris Schmidt: but we and several other people have seen it. Within 15 minutes after you give the drug, there is massive release of

serotonin from the terminals: then over the next 24 hours, there is recovery of 5-HT levels. The cells recover, the axons contain serotonin, and then from 1 to 3 days later, the axons become enormously swollen and fragmented. The sequence is that there is release, depletion, and 1 to 2 days later, degeneration of the terminals, followed by gradual regeneration months later.

Q: So you are saying that the depletion, the long-term depletion, is dependent upon degeneration?

A: The long-term depletion is likely due to degeneration; the short-term depletion is not. We have been able to take hippocampal slices and incubate them-remove them from an animal and incubate them in artificial cerebrospinal fluid. If you treat an animal with PCA or MDMA and remove the brain rapidly, the brain slices are depleted of serotonin; if you then put the slices in an incubation unit, serotonin synthesis occurs and the serotonin becomes restored if you sacrifice the animals at 2 hours, 4 hours, 6 hours, or up to 12 hours after the drug. At about 18 hours you no longer get restoration, and that coincides with the time that you see swollen, abnormal-looking axons. Thus, during the first 12 hours, the axons are intact and recovery of 5-HT is seen. At 18 hours, there is degeneration.

Q: Are you suggesting then that you blocked the initial release and it has no effect upon degeneration?

A (Molliver): No. The two mechanisms are dissociated. Ray Fuller and Chris Schmidt have demonstrated that, and we have also confirmed it. You get initial depletion of transmitter, and then you wait, depending on the drug, up to 6 to 12 hours when you can give an uptake blocker (citalopram or fluoxetine) which prevents degeneration. You get depletion dissociated from degeneration. Presumably the amphetamine derivatives are binding to the 5-HT uptake site; if you displace them, within 6 to 12 hours, depending on whether it is MDA or PCA, you can get substantial protection against toxicity.

COMMENT (Ricaurte): Can I just make a comment referring back to what Dr. Landfield was discussing? While I agree with Mark in his interpretation of the data, I think the questions you raise are superb from the standpoint of the level of evidence that we should demand to arrive at a conclusion that indeed we are dealing with a neurotoxic action. The question that I would have for you is, what would it take to convince you that you are dealing with a neurotoxic drug?

A (Landfield): Having done stains for many years and knowing their variability, I always feel a little uncomfortable with them. What I prefer is EM and semithin sections. You can see every element. The observer then can see every type of cell that is there.

COMMENT (Ricaurte): With regard to amphetamine neurotoxicity, Phil Groves has published EM immunocytochemical evidence demonstrating that amphetamine is toxic to striatal dopamine titers.

COMMENT (Landfield): I think you have to see them actually degenerate. There is another thing that bothers me about this interpretation: The sprouting usually takes place in about 15 days in many systems, If only the terminals are damaged, it is unprecedented to see decreases in neurochemistry for this long, because it is also unprecedented for the terminals to take so long to sprout. So there is something very unusual happening here, very interesting.

COMMENT (Molliver): Actually this sprouting begins at 2 to 4 weeks, in regions close to the cell bodies. But it extends to the cortex only over a period of months.

Q: I also had a question regarding your implication of degeneration and regeneration since you only use a positive stain here-immunochemistry. It would seem to me that you are implying degeneration, but you should use degeneration staining to really show that in the first place. And in the second place, you should really count cells, cell bodies. Because I have done an entire series of MPTP-treated animals, I see the entire phenomenon that you see, including the swellings. But the swellings turn out to be swollen axons that stain with the Fink-Heimer method. However, after a period of time, they are gone; and later still, the cell bodies are *gone,* totally gone. Other studies have shown that it can take up to a year or 18 months for long-axon systems to degenerate all the way. I think that many of your data should be supplemented by some of the silver methods and counting some of the cell bodies.

A (Molliver): I agree with you. We have resisted counting cell bodies,

COMMENT: I understand why.

COMMENT (Seiden): It may be possible that in a highly collateralized system like the 5-HT system, unless you damage some of the collaterals, the cell bodies will not degenerate.

COMMENT (Appel): There are data that I can offer from experiments we did at the Addiction Research Center of NIDA in Baltimore to support what Mark says and might answer some of your criticisms because you asked about other approaches. The approach that we used was in vitro receptor autoradiography. We looked at the 5-HT uptake site. Using paroxetine binding, I did a study after fenfluramine treatment at high dose, and George Battaglia did a study after

MDMA or MDA treatment that is in press now in Synapse. Basically, what the three different protocols showed was a decrease in the density of 5-HT uptake sites throughout the frontal cortex, striatum, hippocampus, our hypothesis being that the uptake site is on the axon. If the axon degenerates, there is no substrate for the uptake site. The uptake site disappeared and was not seen immediately or 2 weeks following treatment. However, there wasn't a change in the density of these uptake sites on the dorsal and medial raphe, the site of the cell bodies from which regeneration could occur later on.

That is one set of studies. It was neurochemically specific, because what we also did (and what George did) was to use mazindol to look at catecholaminergic uptake sites vs. serotonin to show that those sites did not change at all. Rob Zaczek did a study with fenfluramine and long-term recovery (and these are now homogenate assays) looking for serotonin and also serotonin uptake sites with paroxetine binding. The serotonin uptake sites showed a very slow recovery. The study was carried out only to 32 weeks or 8 months. There was a slow recovery; it never reached 100 percent. I think at the 30-week time it was still at about 70 percent for the 5-HT uptake sites. This is just a homogenate of frontal cortex, so it is general. What was interesting was with the 5-HIAA and the serotonin content (these were measured in hippocampus and in frontal cortex). There was a transitory increase in serotonin and 5-HIAA. At 8 weeks, there was a transitory increase in hippocampus; then it dropped back down. At 16 weeks, it was a transitory increase in cerebral cortex, and then it dropped back down. What I was wondering when you were showing these little diagrams is if there could be a corresponding time course, where you have these large bulbs (for lack of a better word)-the transitory appearance of them. Then they disappear, because you start to get sprouting of your smaller fibers, your filopodia. And then you were having your transient increase, or maybe the production that is blocking up at the end of these damaged axons. When regeneration starts occurring, 5-HT starts moving on, and maybe the levels drop back down. Rob's study was done only in fenfluramine-treated animals and was carried out only as far as the 32 weeks. So this problem has been looked at with more than just immunochemistry. I suffered the same "slings and arrows" when I looked at fenfluramine. You are trying to stain for a neurotransmitter that is not there. So there are other approaches.

Q (Seiden): Wasn't there a 64-week study?

A (Appel): I don't think so; there is a MDMA study approaching 1 year.

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[1] ***Synapse* 8(4):249-260, 1991.**

COMMENT (Ricaurte): George Battaglia and Errol De Souza did a 12-month study with MDMA. After the 12 months, the uptake sites were completely recovered. However, serotonin was only recovered to 50 percent of control.

COMMENT (Appel): There are still some rats sitting back at Baltimore; everybody has left, but there are still a couple of rats. (Laughter)

COMMENT (Landfield): I think that it is clear that there are major neurochemical effects of these treatments. But I still would like to see some more done to directly visualize degenerating serotonergic synapses and cells.

COMMENT (O'Callaghan): Perhaps with a good TPH [tryptophan hydroxylase] antibody, one could stain the terminal fields that are degenerating. In the early stages of a loss of immunoreactivity of serotonin in that terminal field, could you double label with neurofilament antisera and see the progression of loss?

COMMENT (Molliver): Yes, you could. We have tried that. The problem is that fine, unmyelinated axons are very few and contain little or no neurofilament protein.

Q (Cho): Has anybody studied those with EM?

A (Molliver): We have tried to. The problem is that once you get to sections that are a fraction of a micron thick, the incidence of affected axons is quite small. There actually is one report recently by Constantine Sotello in Paris who has shown EM evidence of degenerating axons after fenfluramine.

Q (Seiden): What is the best alternative hypothesis?

A (Landfield): The best alternative hypothesis I would think is that there is something about the uptake of these drugs that blocks axon transport of the transmitter serotonin, so that you have a loss of transmitter in terminals for 1 or 2 months; at least the synapses are nonfunctional. Something like that would account for why there is no loss of serotonin all the way back in the cell body. What are the uptake sites? They are proteins, which may be altered by treatment. They may be phosphorylated, or changed in conformation so that they may not bind. There could be all sorts of changes in uptake or transport mechanisms.

COMMENT (Seiden): It is not only the binding aspect of serotonin uptake sites that is compromised. If you do the synaptosomal preparation and you look at the uptake of labelled serotonin, you will find that there is a change in B_{max}.

COMMENT (Landfield): I am not arguing that that is not true. I just saw a paper where calbindin immunostaining totally disappeared after 15 minutes of exposure to calcium. The treatment simply changed the conformation of the protein so it was no longer visible by immunostaining. So one could imagine many ways to inactivate binding to channels or uptake sites.

COMMENT (Molliver): Calbindin was found in the cell bodies of calbindin-positive and GABA [gamma aminobutyric acid] cells. When their axons are transected close to the cell body, the cells stop producing transmitter-synthesizing enzymes. In this study, the raphe cells are still producing transmitter-synthesizing enzymes, which are accumulating along the axon. That is not a comparable situation.

COMMENT (Landfield): I am just using that as an example of the sensitivity of immunostaining and how it can change, just like that.

COMMENT (Appel): I can offer you still another approach which is going on right now. We have treated some animals with fenfluramine or saline, let them recover for 2 weeks, then injected tritiated proline into their dorsal raphe, and are looking for anterograde transport or the lack of transport. I guess what we are looking for is not so much a smoking gun as a gun with a stuffed muzzle. I can tell you that I developed the injection sites-all the animals received their tritiated proline and a first set of films has been developed after 6 weeks. It is going to take a lot longer than 6 weeks to know the results, but here is still another approach, and this type of approach is not based on the neurotransmitter-it is based on the locus from which regeneration occurs.

Q (Landfield): I think that's a good approach.

Q (Heimer): I was thinking why not try PHAL method.

A (Appel): I didn't have an iontophoretic apparatus.

Q (Heimer): Using the PHAL method, you will be able to see the morphology of these fibers very nicely, which you can't do in autoradiography.

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Quantification of Reactive Gliosis as an Approach to Neurotoxicity

James P. O'Callaghan and Diane B. Miller

INTRODUCTION

If handed a bottle containing a pure chemical, told its molecular structure and melting or boiling point, and then asked whether this substance was neurotoxic, most scientists probably would not even hazard a guess. However, the U.S. Environmental Protection Agency (EPA) must face this "real world" scenario on a daily basis. For example, under the Toxic Substances Control Act (15 USC 2601 et seq. 1976), the EPA's Pre-Manufacturing Notification Process requires companies to give notification of their intent to manufacture a new chemical or mixture. Within 90 days of receipt of this information, the EPA must decide whether there are sufficient grounds to deny permission to manufacture the compound. This decision is to be based on "the potential risk of injury to health or the environment" posed by exposure to the compound (15 USC 2601 et seq. 1976). Ideally, risk of injury to health posed by exposure to a given compound should include not only the effects linked to the most widely known "icities," carcinogenicity and teratogenicity, but neurotoxicity as well. In practice, however, it is unlikely that the compound's potential to cause nervous system damage would be considered, because there is no widely accepted means for assessing neurotoxicity. Thus, in the absence of prior knowledge of the potential of a given chemical or mixture to produce neurotoxic effects, there would be no reason to bar its production. This is a problem not just for the introduction of new chemicals. Of the 65,000 chemicals in EPA's existing chemical inventory, few have been screened for neurotoxicity (National Research Council 1983; U.S. Congress 1990; Tilson 1990). Clearly, from the perspective of the EPA, the sheer volume of potentially neurotoxic substances dictates the need for development and application of broadly applicable approaches for assessing neurotoxicity. The purpose of this chapter is to show how the application of one such approach, quantification of reactive gliosis, can be used to assess the neurotoxic effects of broad classes of chemicals found in the environment. In this context, drugs of abuse will be considered as "environmental pollutants" to illustrate how this approach can be extended to compounds of interest to another Federal agency, the National Institute on Drug Abuse (NIDA).

Why is assessing neurotoxicity so difficult? A major reason is that targets of neurotoxic insult are diverse and unpredictable. In comparison to other organ systems, the mammalian central nervous system (CNS) is extremely complex. It is composed of a vast array of different types of neurons and glia. At the molecular level, this cell-type heterogeneity is reflected in the diversity of distinct brain mRNAs (messenger RNAs) and their respective proteins. As many as 30,000 mRNAs are thought to exist, and 20,000 of these may be brain specific (Sutcliffe 1988). These diverse cell types provide potentially equally diverse targets of chemical-induced injury. Indeed, the classic textbook on experimental neurotoxicology by Spencer and Schaumburg (1980) documents the multiplicity of "selectively vulnerable" targets of neurotoxicity. As exemplified by the dilemma faced by the EPA, often there is no database that points to the targets affected by a suspect neurotoxicant. It seems obvious that this situation has led neurotoxicologists to rely heavily on morphological evaluations for assessing neurotoxicity (for a discussion, see Switzer 1991; Beltramino et al., this volume).

Morphological measures undeniably remain the "gold standard" for neurotoxicity assessments. Without prior knowledge of the actions of a given compound, morphologically based approaches serve as the only means for locating affected regions and cell types. Moreover, because morphological changes generally are taken as evidence of structural alterations, they often are thought to be indicative of irreversible damage or, at the least, of a pathological process. Therefore, it is not surprising that chemical-induced changes in nervous system morphology are widely regarded as being indicative of an undesirable and, hence, adverse or "neurotoxic" effect. It can be argued that biochemical, physiological, and behavioral endpoints also can be used as indicators of neurotoxicity. Should permanent changes in any of these measures occur as a result of chemical exposures, they could be taken as indications of adverse (i.e., neurotoxic) effects on the nervous system. Reversible changes in these measures are more difficult to interpret. Although they could be indicative of damage to specific brain regions or cell types, such changes instead may reflect reversible pharmacological actions of the compound being studied. As noted by Switzer (1991), it is not sensible to describe the change in a particular endpoint without determining whether that change is related to destruction or damage of the anatomical entity that produces or controls that endpoint. This does not imply that reversible pharmacological effects should not be considered adverse or perhaps even neurotoxic (Tilson 1990). Nevertheless, it seems likely that a particular endpoint will gain broader acceptance as an indicator of neurotoxicity if it can be linked to neural damage, given that nervous system damage is almost universally accepted as an adverse effect. If neural damage is to be taken as the most common basis for defining the neurotoxic state, then it follows that there should be a set of criteria for defining this condition. Such criteria do indeed exist.

CRITERIA FOR DEMONSTRATION OF NEURAL DAMAGE

There are at least three widely accepted morphological criteria for establishing the presence of neural damage. They are (1) cell loss, (2) argyrophilia (silver staining), and (3) gliosis. Demonstration of any of the three is presumptive evidence for neural damage. In the context of this chapter, the term "damage" does not necessarily connote cell destruction. Thus, argyrophilia and gliosis (criteria 2 and 3) can be used as indicators of damage in the the absence of evidence for cell loss (criterion 1).

In theory, application of any of the above three criteria to neurotoxicity assessment will aid in identifying damaged neural targets. In practice, although application of the first criterion may be suitable for determining chemical-induced cell loss in regions with large cell types organized in easily recognized layers (e.g., hippocampus and cerebellum), damage to other vulnerable regions may go undetected (O'Callaghan and Jensen 1992; Beltramino et al., this volume). Only recently have unbiased cell-counting methods been developed in an attempt to overcome this problem (Moller et al. 1989). The second criterion, an argyrophilic reaction to neural damage, has proven to be an earlier and more sensitive indicator of the neurotoxic condition (Balaban et al. 1988; Switzer 1991; O'Callaghan and Jensen 1992; Balaban 1992; Beltramino et al., this volume). Moreover, unlike the classic basic dyes used to detect cell loss (e.g., cresyl violet and H and E), silver stains can reveal damage to nerve terminals as well as damage or loss of neuronal perikarya (Balaban et al. 1988; O'Callaghan and Jensen 1992; Beltramino et al., this volume). The use of silver degeneration stains in assessments of neurotoxicity is reviewed elsewhere in this monograph by Beltramino and colleagues and by Jensen and colleagues. The focus of the remainder of this chapter is on the application of the third criterion, gliosis, for assessing toxicant-induced injury of the CNS.

APPLICATION OF GLIAL FIBRILLARY ACIDIC PROTEIN ASSAYS TO DETECT AND QUANTIFY GLIOSIS

Convinced of the need for a broadly applicable approach to neurotoxicity assessment linked to neural damage, the authors and colleagues wanted to utilize an endpoint that would reveal damage anywhere in the CNS following exposure to any type of neurotoxicant. This endpoint needed to be a sensitive indicator of neural damage as well as one that could be readily quantified. Gliosis, a condition otherwise known as astrogliosis or reactive gliosis, satisfies all these requirements.

Reactive gliosis occurs following nervous system damage induced by a variety of insults, including physical damage, disease, or chemicals (Eng 1988; O'Callaghan 1991 a, 1991*b*, 1992; Hatten et al. 1991; Norton et al. 1992). It is characterized by hypertrophy and, rarely, hyperplasia of astrocytes, a subtype

of CNS glia (O'Callaghan 1991*a*, 1991*b*; Norton et al. 1992). Damage to any neuronal and glial cell type (including astrocytes themselves) appears to elicit reactive gliosis anywhere in the CNS. Classically, reactive astrocytes were distinguished by their increased size, longer and thicker processes, and increased content of glial filaments (Norton et al. 1992).

The development of antibodies to glial fibrillary acidic protein (GFAP), the major protein of astrocyte intermediate filaments (Eng 1985) has led to the widespread application of GFAP immunocytochemistry for studying reactive gliosis (Lindsay 1986; Eng 1988). Although the results of these studies have firmly established gliosis as a dominant response to traumatic injury of the CNS, only a few investigations have been devoted to an examination of gliosis following chemical exposures (O'Callaghan 1991*a*, 1991*b*, 1992; O'Callaghan and Jensen 1992; Norton et al. 1992). Moreover, most attempts to quantify gliosis have been limited to counts of GFAP-positive cells in immunostained sections, Not only is this approach tedious, but it is hampered by a lack of sensitivity as well as fixation artifacts (Shehab et al. 1990; Norton et al. 1992). Because antibodies to GFAP are widely available through commercial sources, it was reasoned that development of a GFAP radioimmunoassay offered the most viable option to achieve sensitive and rapid quantification of GFAP as an indicator of the degree of reactive gliosis.

The authors have developed two assays for GFAP: a solid phase immunoassay (O'Callaghan and Miller 1985; Brock and O'Callaghan 1987) and a sandwich enzyme-linked immunosorbent assay (ELISA) (O'Callaghan 1991*c*). The former procedure is a modification of the nitrocellulose-based, dot-immunobinding procedure of Jahn and colleagues (1984). The latter is a novel microtiter plate assay employing both monoclonal and polyclonal antibodies to GFAP in a sandwich format (O'Callaghan 1991 c). Both procedures share the advantage that they can be used to assay detergent homogenates of nervous tissue directly without further fractionation or purification. Thus, the data obtained are indicative of effects that occurred in intact tissue, not in an extract of an operationally defined membrane or cytosol fraction. It is likely that the sandwich ELISA will prove to be more widely applicable than the dot-immunobinding procedure because it is cheaper and faster and does not require the use of radioactive reagents (O'Callaghan 1991*c*). When both GFAP assays are used to analyze the same samples, essentially identical results are obtained (O'Callaghan 1991*c*).

VALIDATION OF GFAP AS A BIOCHEMICAL INDICATOR OF NEUROTOXICITY

If reactive gliosis is a common response to neural damage, homogenates of brain regions damaged by known neurotoxicants should contain an increased concentration of GFAP. To test this hypothesis, the authors examined the effects

of prototype neurotoxicants known to damage diverse cell types in different regions of the CNS. Both adult and developing rodents were used in these validation experiments. The results of these studies have been reviewed (O'Callaghan 1988, 1991*a*, 1991*b*, 1992; O'Callaghan and Miller 1989; O'Callaghan and Jensen 1992). Quantitative increases in GFAP that result from brain injury induced by agents or conditions other than those employed in our investigations also have been reported. These studies have been reviewed (Hatten et al. 1991; Norton et al. 1992).

Some of the prototype neurotoxicants employed, all of which resulted in an increase in GFAP, were trimethyltin (TMT), kainate, triethyltin, ibotenate, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, bilirubin (Gunn rat), 5,7-dihydroxytryptamine (5,7-DHT), cadmium, colchicine, 3-acetylpyridine, methylmercury, methamphetamine, iminodipropionitrile, methylazoxymethanol, and ethanol. Compounds chosen were known to damage neurons in several different brain regions to determine whether astrocyte responses could be observed in response to injury of any area of the CNS. Compounds also were chosen that ranged from those that caused overt cell loss (e.g., TMT and bilirubin) (Brock and O'Callaghan 1987; O'Callaghan and Miller 1985) to those that caused damage to subcellular elements that would not be detected in routine neuropathological examinations. An example of a compound in the latter category is MPTP, a substituted pyridine known to damage dopaminergic nerve terminals (Hallman et al. 1985; Heikkila et al. 1984). In our validation scheme, we first emphasized studies of compounds that caused outright cell loss. Overt neuron loss manifested at the light microscopic level is not a subtle effect. Therefore, if large increases in GFAP were not seen with compounds in this category, the utility of GFAP as an indicator of neural damage would be limited. We were not disappointed. Compounds that caused readily recognizable destruction of neuronal perikarya, such as TMT (Brock and O'Callaghan 1987), resulted in large (as much as 6,000 percent) increases in GFAP in the affected brain region (Brock and O'Callaghan 1987). The results of these initial studies provided the impetus to examine the effects of compounds where damaged neural components (as revealed by a permanent loss of a specific marker protein) would not be evident from Nissl staining (e.g., MPTP). Here, too, large increases in GFAP (350 percent of control) were found in brain regions known to be damaged by the compound being used (O'Callaghan et al. 1990*a*, 1990*b*).

A number of features associated with chemical-induced gliosis emerged from our studies of prototype neurotoxicants. A review of these features illustrates the advantages and limitations of the use of GFAP as a biochemical indicator of neural damage. A list and brief synopsis of our observations follows:

1. Large, dose-dependent increases in GFAP result from acute exposure to chemicals that destroy neuronal perikarya. Thus, increases in GFAP occur under conditions associated with outright cell loss as evidenced by loss of Nissl staining.

2. Large, dose-dependent increases in GFAP result from acute exposure to chemicals that damage cellular components not revealed by Nissl staining. Thus, assays of GFAP are sensitive enough to reflect damage to cellular elements under conditions where there is no Nissl-staining evidence of cell death or damage, a level of sensitivity shared by reduced silver methods for detecting neuronal degeneration (O'Callaghan and Jensen 1992; Beltramino et al., this volume; Jensen et al., this volume).

3. Targets of chemical-induced neural damage are revealed by increases in GFAP. Trauma-induced gliosis can spread to an entire brain hemisphere following a stab lesion (Mathewson and Berry 1985). In contrast, chemical-induced lesions appear to be confined to the site of damage (Balaban et al. 1988; O'Callaghan 1991*a*, 1991*b*, 1992). Thus, assays of GFAP should be useful for localizing sites of chemical-induced brain damage. However, this will be the case only if the increase in GFAP is large enough to be detected within the sample of tissue subjected to assay. Ensuring that toxicant-induced increases in GFAP are not masked by the baseline concentration of GFAP in unaffected tissue requires dissection of the brain into several regions. Dividing the brain into six to eight regions (O'Callaghan 1991*b*) has proven sufficient to locate areas of gliosis. To reiterate, assays of GFAP will not reveal sites of reactive gliosis if such sites represent only a small percentage of the total tissue sampled.

4. Chemical-induced increases in GFAP are not permanent. The astrocyte response to traumatic injury or disease often is manifested by a persistent glial scar that is GFAP positive (Reier 1986). In contrast, we found that all chemical-induced injuries of the CNS examined to date result in an increase in GFAP that abates with time. The rate of onset and the subsequent decline in GFAP levels vary markedly from toxicant to toxicant (O'Callaghan 1991*a*, 1991*b*; Norton et al. 1992). Some chemicals cause an increase in GFAP that is slow to develop and slow to decline. For example, with TMT the peak increase does not occur until 5 weeks postdosing, and the return to baseline level is not reached until 12 weeks (Brock and O'Callaghan 1987). Other chemical injuries result in a rapid onset and a rapid decline in GFAP. For example, with MPTP the peak increase occurs within 48 hours and the return to control occurs by 21 days (O'Callaghan et al. 1990*a,* 1990*b*). The reason for these disparate time-effect relationships remains unknown. Our current hypothesis is that sustained cellular damage is required to maintain a continued elevation of GFAP (see O'Callaghan et al. 1990*a* for a discussion of this issue). Sustained damage may reflect the continued presence of the

toxic insult (O'Callaghan et al. 1990*a*) or, as emphasized by Heimer and coworkers (Beltramino et al., this volume), a "cascading effect" involving pathways "recruited into the pathophysiologic process." Regardless of the mechanisms responsible for induction and maintenance of the GFAP response to chemical-induced injury, our findings indicate that assessments of neurotoxicity using assays of GFAP should employ multiple time points to prevent false-negative results.

5. Hypertrophy, not hyperplasia, is the dominant response to chemical-induced injuries of the CNS. Early studies by Cavanagh (1970) suggested that astrocytes divide in response to penetrating injuries of the CNS. This hyperplastic response is purported to be the basis of the glial scar (Reier 1986). A study using [^{3}H]thymidine autoradiography combined with GFAP immunocytochemistry confirmed that stab injuries cause astrocytes to divide in the region surrounding the wound site (Latov et al. 1979); this study often serves as the basis of the contention that massive astrocytic hyperplasia accompanies all brain injuries. However, careful examination of the data indicates that stab injuries result in astrocyte proliferation only in the vicinity of the wound site (Latov et al. 1979; Hatten et al. 1991; Norton et al. 1992). Moreover, even at the site of injury, only a small percentage of astrocytes proliferate (Latov et al. 1979). Both at the wound site and especially in the surrounding tissue, hypertrophy is the dominant astrocytic reaction (Norton et al. 1992). In studies of prototype neurotoxicants, the authors confirmed that astrocytic proliferation can occur (Brock and O'Callaghan 1987) but, again, only on a limited basis. Less than 1 percent of total astrocytes were found to undergo division (Brock and O'Callaghan 1987). Given that blood contains astrocyte mitogens (Giulian et al. 1988) it is not surprising that astrocytes divide in the vicinity of stab wounds. However, when the blood-brain barrier remains intact, as is the case following exposure to MPTP (O'Callaghan et al. 1990*a*), large increases in GFAP occur apparently without the proliferation of astrocytes (Brock and O'Callaghan 1987; O'Callaghan 1991*a*). If our data for MPTP and other prototype neurotoxicants can be applied to the general case, we do not expect astrocytic hyperplasia to play a major role in the observed increase in GFAP. Given this probability, it is doubtful that GFAP immunocytochemistry-based cell counts will be of much value in the assessment of general neurotoxic responses because more GFAP per astrocyte, not more astrocytes, will be the predominant neurotoxic response.

6. Pharmacological agents at therapeutic dosages do not cause an increase in GFAP. One of the assumptions underlying the authors' use of GFAP assays to detect and quantify neural damage is that drugs administered at therapeutic dosages would not cause an increase in GFAP. Although comprehensive dose- and time-effect studies have not been attempted, compounds from several pharmacological classes have been examined. None of these agents affected GFAP levels in any brain region examined

(O'Callaghan 1991*a*, 1991*b*, 1992). These findings are consistent with our view that increases in GFAP occur as a consequence of chemical-induced neural damage but do not result from drug exposures at therapeutic dosages. This does not mean that drug dosages above the pharmacological range would not result in neural damage (see below).

In aggregate, these findings indicate that assays of GFAP can be used to quantify dose-, time-, and region-dependent patterns of toxicant-induced astrogliosis (O'Callaghan 1991*a*, 1991*b*). Using this response as an indirect indicator of chemical-induced neural damage, we have been able to detect effects at toxicant dosages below those that would result in light microscopic evidence of neurotoxicity. As expected, pharmacological agents at therapeutic dosages do not appear to elicit this generalized response to nervous system damage.

APPLICATION OF GFAP ASSAYS TO DETECT AND QUANTIFY THE NEUROTOXIC EFFECTS OF SUBSTITUTED AMPHETAMINES

Various substituted amphetamines (e.g., amphetamine, methamphetamine, methylenedioxyamphetamine, methylenedioxymethamphetamine [MDMA], fenfluramine, parachloroamphetamine) are considered to be neurotoxic because they produce long-term (several days to several months) decrements in indicators of brain dopaminergic and serotonergic function (Fuller 1989, 1978, 1985; Seiden and Kleven 1989; Gibb et al. 1990; Molliver et al. 1990; Schmidt and Kehne 1990; De Souza et al. 1990). Included among these effects are decreases in brain amines (Ricaurte et al. 1980, 1984*a*; Wagner et al. 1980; Commins et al. 1987; Schmidt 1987), decreases in the rate-limiting enzymes responsible for amine biosynthesis (Hotchkiss and Gibb 1980; Stone et al. 1991; Schmidt and Taylor 1987; Lovenberg and Bruckwick 1978), and decreased ligand binding to high-affinity uptake sites for dopamine and serotonin (Commins et al. 1987; Appel et al. 1989; Kovachich et al. 1989; Schmidt 1987; Battaglia et al. 1987). At higher dosages of these compounds, evidence of neural damage has been observed using silver degeneration stains (Commins and Seiden 1986; Commins et al. 1987; Ricaurte et al. 1982, 1984*a*, 1984*b*; Scallet et al. 1988; Jensen et al., this volume). In the examination of these compounds, the intent was to establish whether the previously observed effects on dopaminergic and serotonergic systems were accompanied by the astrocytic (GFAP) response characteristic of neural damage. Where an increase in GFAP was observed, a related goal was to establish whether damaged dopaminergic or serotonergic neurons served as a stimulus for this effect. Silver degeneration stains also were employed as an alternate means of detecting sites of damage (see Jensen et al., this volume). Two amphetamine derivatives, methamphetamine and MDMA, were evaluated. These compounds were chosen because they represent prototype amphetamines for producing long-term deficits in dopaminergic and serotonergic systems, respectively (Gibb et al. 1990).

EFFECTS OF MPTP AND METHAMPHETAMINE IN THE C57BL/6J MOUSE

Recently, Sonsalla and colleagues (1989) compared the effects of MPTP and methamphetamine on the nigrostriatal dopaminergic system of the C57Bl/6J mouse. Both compounds produced deficits in dopamine levels and in the activity of tyrosine hydroxylase (TH), a marker enzyme of the nigrostriatal dopaminergic pathway. These effects were equated with the "neurotoxic" actions of these compounds. Pretreatment with the noncompetitive glutamate receptor antagonist, MK-801, blocked the effects of methamphetamine but not those of MPTP.

From previous work on MPTP in the mouse, it is known that long-term decrements in striatal dopamine are accompanied by apparently permanent decrements in the concentration of TH holoenzyme protein (O'Callaghan et al. 1990*a*, 1990*b*). Moreover, a large (350 percent of control) increase in GFAP is associated with the loss of TH protein (O'Callaghan et al. 1990*a*) as is an argyrophilic reaction in the nigrostriatal pathway (O'Callaghan and Jensen 1992). Taken together, these data are viewed as strong evidence for a neurotoxic action of MPTP on nigrostriatal dopaminergic neurons. Thus, MPTP-induced depletion of striatal dopamine in the mouse can be considered a neurotoxic effect, because this action of the compound is associated with changes in other endpoints that are indicative of neural damage. The effects of methamphetamine on striatal dopamine in the mouse may also be indicative of damage to this pathway, but indicators of neural damage, such as loss of TH protein or an increase in GFAP, have not been examined. To address this question, we adopted the methamphetamine-dosing regimen for the mouse that was developed by Sonsalla and coworkers (1989). MPTP served as a positive control and, as in the study by Sonsalla and colleagues (1989) the potential neuroprotective effects of MK-801 were evaluated.

As demonstrated previously (O'Callaghan et al. 1990*a*), a single subcutaneous (SC) injection of MPTP (12.5 mg/kg) results in a large (300 percent of control) increase in striatal GFAP at 2 days postdosing (figure 1). A greater-than-50-percent reduction in the concentration of TH (protein) and dopamine accompanies the increase in GFAP. None of these effects of MPTP were altered by pretreatment and posttreatment with MK-801 (2x1 mg/kg; see Sonsalla et al. 1989). Administration of methamphetamine (4x5 mg/kg in 1 day; see Sonsalla et al. 1989), like administration of MPTP, caused a large increase in striatal GFAP at 2 days postdosing (figure 2). A greater-than-50-percent reduction in the concentration of TH (protein) and dopamine accompanied the increase in GFAP. In contrast to the effects of MPTP, the methamphetamine-induced increase in GFAP and the decrease in TH and dopamine were completely antagonized by MK-801. All the effects of MPTP and methamphetamine observed at 2 days postdosing also were observed at a 7-day time point (data not shown). These data are in complete agreement

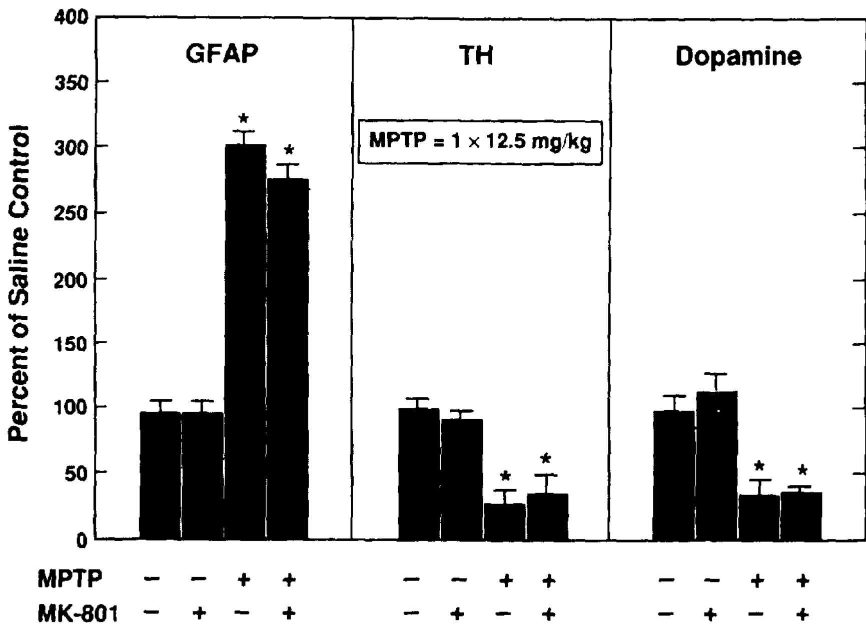

FIGURE 1. *Effects of MPTP alone or in combination with MK-801 on the concentration of GFAP, TH (protein), and dopamine in striatum of C57Bl/6J mice. Mice received 12.5 mg/kg MPTP SC and were killed at 48 hours postdosing. MK-801 (2x1 mg/kg) was administered according to* the *regimen described by Sonsalla and colleagues (1989). GFAP, TH, and dopamine were determined as described in O'Callaghan* (1990*a*).

* Significantly different from corresponding control, $p<0.05$

with those of Sonsalla and colleagues (1989). We conclude that both MPTP and methamphetamine damage nigrostriatal dopaminergic neurons in the mouse and that the effects of the latter compound may be mediated through a glutaminergic mechanism.

EFFECTS OF METHAMPHETAMINE IN THE LONG-EVANS RAT

Methamphetamine is viewed as a dopaminergic neurotoxicant in the rat as well as in the mouse. In comparison to the mouse, however, rats require considerably higher dosages to achieve equivalent depletions of striatal dopamine or reductions in TH activity (Sonsalla et al. 1986). In an attempt to achieve decrements in striatal dopamine in a time equivalent to that of the mouse model, we administered four dosages of 50 mg/kg SC to the Long-Evans

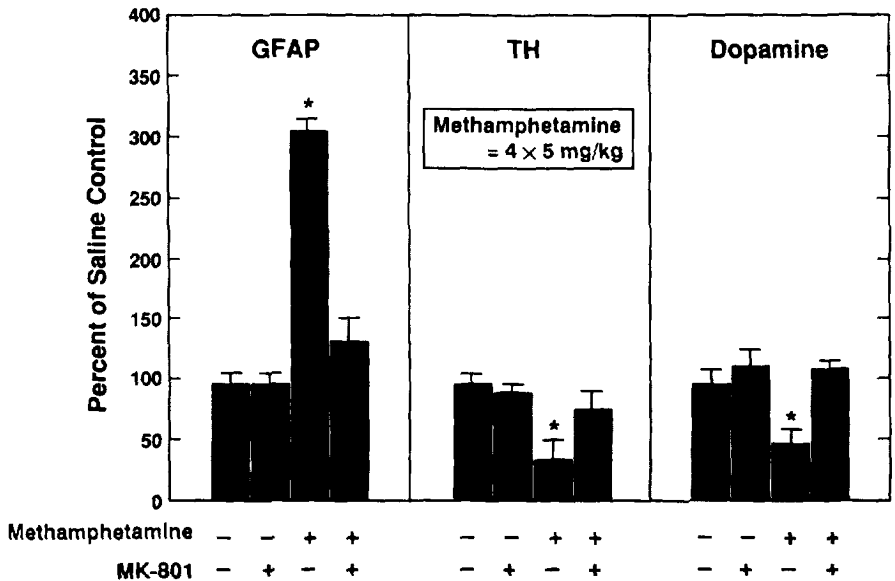

FIGURE 2. *Effects of methamphetamine* alone *or in combination with MK-801 on the concentration of GFAP, TH (protein), and dopamine in striatum of C57Bl/6J mice. Mice received 5 mg/kg methamphetamine, 4 times in 1 day SC and were killed at 48 hours postdosing. The methamphetamine dosing regimen* was as *described by Sonsalla and colleagues (1989). MK-801 (2x1 mg/kg) was administered according to the regimen described by Sonsalla and colleagues (1989). Measurements* were determined as described in the *legend to* figure 1.

* Significantly different from corresponding control, $p<0.05$

rat on a schedule identical to that used for the mouse studies, To prevent mortality with this high-dose regimen, rats were housed individually in wire-bottom cages with a 1 -week acclimation period (D.B. Miller and J.P. O'Callaghan, unpublished observation). As in the mouse studies, we also evaluated the neuroprotective effect of MK-801. At 2 days postdosing, the concentration of striatal GFAP was slightly elevated; however, the concentration of TH was unaffected (figure 3). In contrast, the concentration of striatal dopamine was reduced by more than 50 percent, that is, to levels equivalent to those seen in the mouse at 1/10 the dosage. MK-801 pretreatment and posttreatment (2x1 mg/kg) SC did not alter the effects of methamphetamine on GFAP and dopamine (figure 3). The effect of methamphetamine on striatal dopamine was transient; at 7 days postdosing, dopamine levels had returned to control values (data not

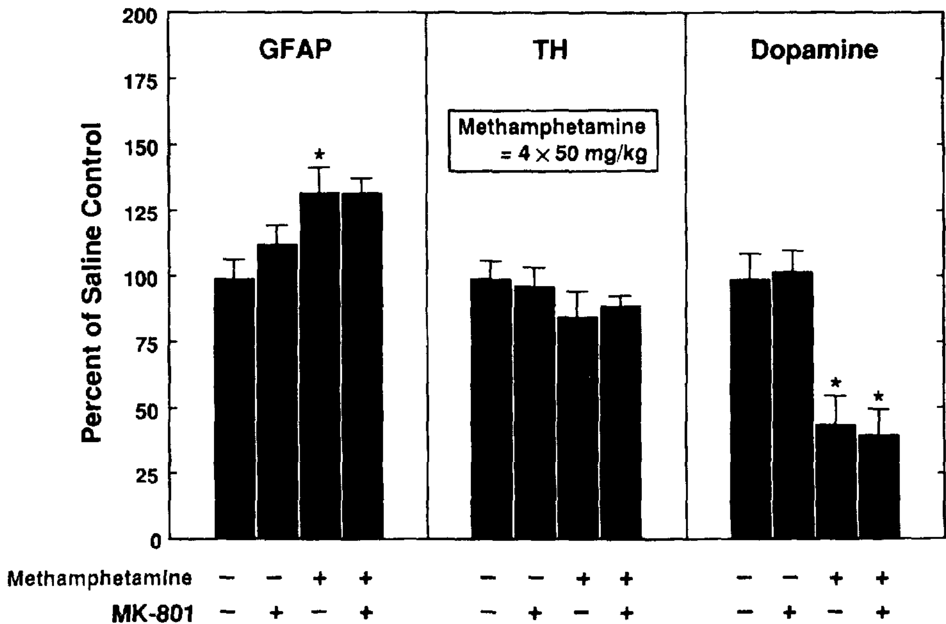

FIGURE 3. *Effects of methamphetamine alone or in combination with MK-801 on the concentration of GFAP, TH (protein), and dopamine in striatum of Long-Evans rats. Rats received 50 mg/kg methamphetamine, 4 times in 1 day SC and were killed at 48 hours postdosing. The dosing schedules for methamphetamine and MK-807 (2x1 mg/kg) were the same as used for the mouse experiments (figure 2). Measurements were determined as described in the legend to figure 1.*

* Significantly different from corresponding control, $p<0.05$

shown). At the 7-daytime point, the concentration of GFAP and TH also did not differ between the control and methamphetamine groups (data not shown).

Our findings suggest that the mouse and rat respond in different manners to methamphetamine. By comparing the GFAP and TH data obtained from the two species, it would not be speculative to conclude that methamphetamine is, at best, weakly neurotoxic to rat nigrostriatal neurons. At the least, our findings suggest that the transient methamphetamine-induced decrease of striatal dopamine in the rat is not an adequate predictor of underlying damage to nigrostriatal neurons. Indeed, the transient effects of methamphetmine on striatal dopamine in the rat may be more of a reflection of the influence of environmental temperature (Bowyer et al. 1992) rather than an indication of

neuronal damage. The fact that an effect of methamphetamine on GFAP and TH in the rat was not observed cannot be viewed as a failure of these endpoints to reveal underlying damage to the nigrostriatal pathway. Intracisternal administration of the dopaminergic neurotoxicant, 6-hydroxy (6-OH) dopamine, to the rat results in a large increase in striatal GFAP (250 percent of control) and a large and permanent decrease in striatal TH protein (J.P. O'Callaghan and G.R. Breese, unpublished observation). Thus, loss of TH protein in association with an increase in striatal GFAP appears to provide an adequate reflection of nigrostriatal damage in the rat. None of our data rule out the possibility that methamphetamine-induced brain damage in the rat may be obtained with dosing regimens that induce long-term decrements in the transmitter content of specific neuronal pathways (Ricaurte et al. 1980). However, even when the methamphetamine dosage was increased to as much as 150 mg/kg, twice daily for 2 days, we failed to see marked increases in GFAP at time points ranging from 2 to 9 days postdosing (data not shown).

EFFECTS OF MDMA IN THE LONG-EVANS RAT

MDMA is widely viewed as being neurotoxic to serotonergic neurons (for reviews, see McKenna and Peroutka 1990, 1991). As little as a single administration of 20 mg/kg to the rat results in long-lasting (several weeks) decreases in 5-hydroxytryptamine (5-HT) levels and tryptophan hydroxylase activity in the rat cerebral cortex (Schmidt 1987; Schmidt and Taylor 1987). Long-term decrements in the density of ^{3}H-paroxetine-labeled 5-HT uptake sites, findings suggestive of serotonergic nerve terminal damage, also have been observed with MDMA after repeated administration of 20 mg/kg, twice daily for 4 days (Battaglia et al. 1987). Multiple high-dose regimens of MDMA result in evidence of terminal degeneration based on reduced silver degeneration stains (Commins et al. 1987). Most of these data have been obtained from studies in the rat because serotonergic neurons apparently are not affected by MDMA in the mouse (Stone et al. 1991; McKenna and Peroutka 1991).

We began our examination of the effects of MDMA by administering a single 20 mg/kg dose. Cortex, striatum, and hippocampus, regions known to show 5-HT depletion with this dose of MDMA, then were assayed for GFAP. Levels of GFAP were not changed at time points ranging from 4 hours to 4 weeks postdosing (figure 4). Because the degree of neurotoxicity associated with the administration of MDMA is purported to be related to the number as well as the size of the doses received (McKenna and Paroutka 1991), we reasoned that a multiple-dose regimen might be required to see an MDMA-induced increase in GFAP. Therefore, twice daily administrations of 5 to 30 mg/kg of MDMA were given for 7 days, and GFAP and 5-HT levels in cortex, hippocampus, and striatum were assayed. The data obtained at 2 days after the last dose of MDMA are shown in figure 5. Again, no increase was seen in GFAP in any of these regions, although large decrements in 5-HT were found. GFAP also was

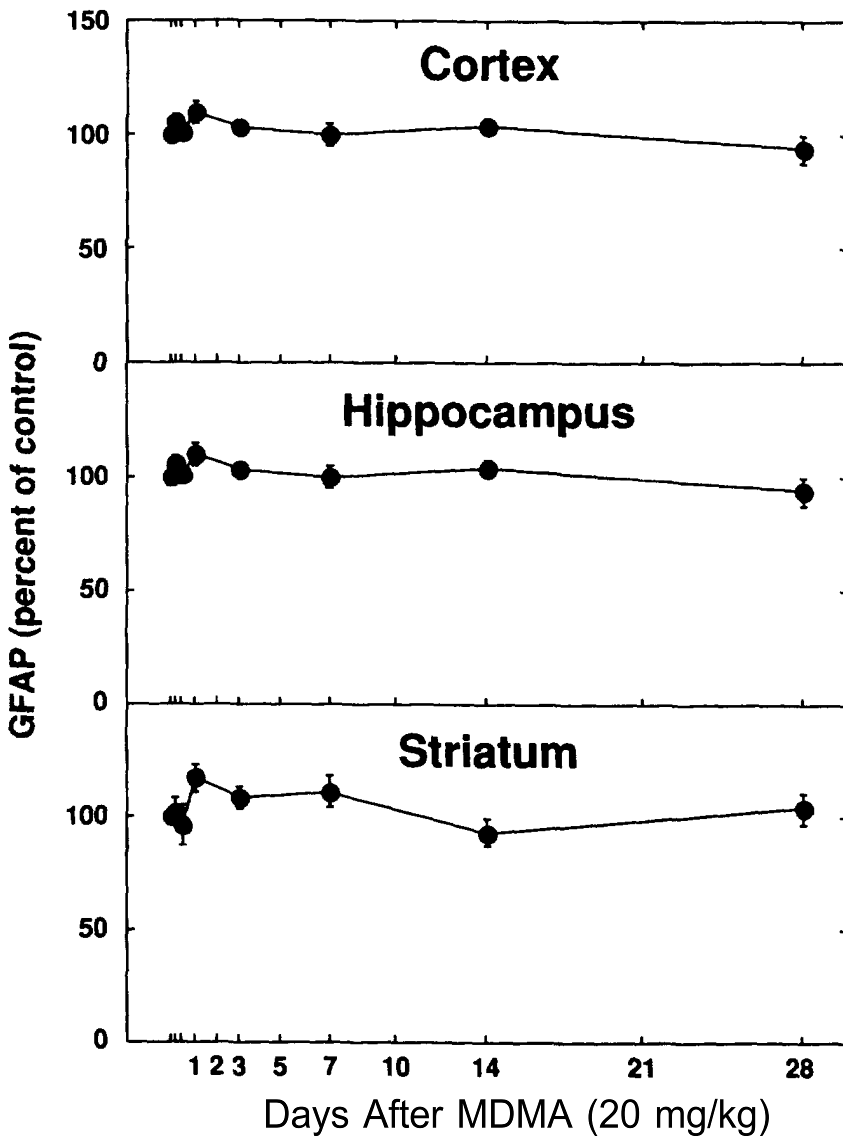

FIGURE 4. *Effects of a single administration of MDMA (20 mg/kg SC) on the concentration of GFAP in cortex, hippocampus, and striatum of the Long-Evans rat. GFAP was determined as described in the legend to figure 1.*

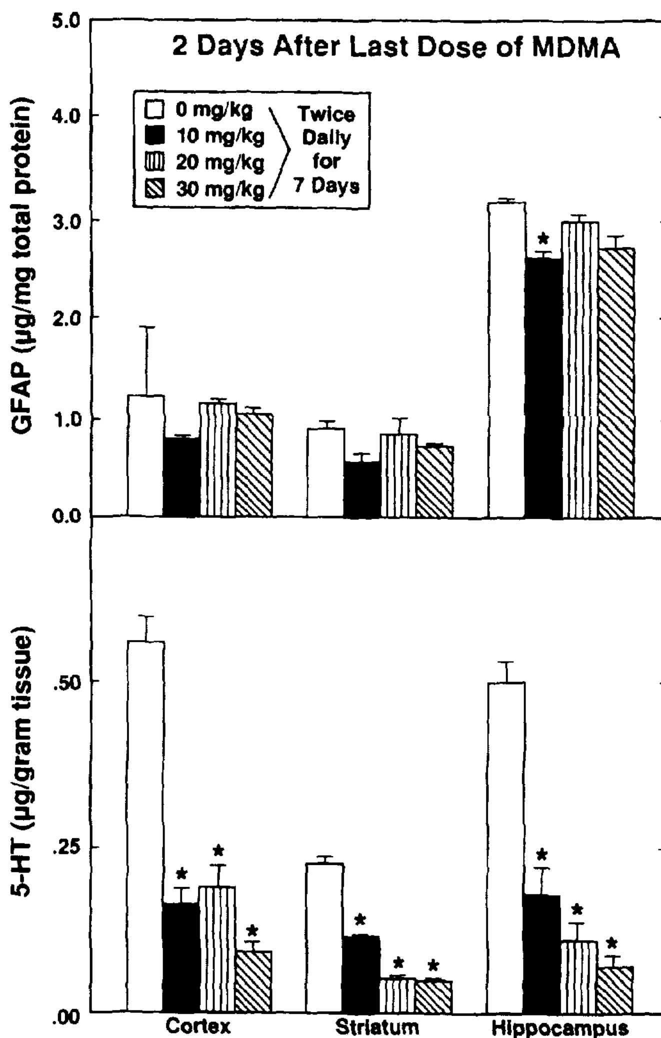

FIGURE 5. *Effects of MDMA (10 to 30 mg/kg, twice daily for 7* days) *on the concentration of GFAP and 5-HT in cortex, striatum, and hippocampus of the long-Evans rat. Rats were killed 2 days after the* last *dose of MDMA. GFAP and 5-HT data were generated from samples prepared from the same rats. GFAP was determined as described in the legend to figure 1. 5-HT was determined by high-performance liquid chromatography (HPLC) coupled with in-line fluorescence detection.*

* Significantly different from control, $p<0.05$

not affected at 7 or 60 days postdosing (data not shown). 5-HT levels, however, remained reduced in striatum and hippocampus but not in cortex at 60 days postdosing (data not shown). These data indicated that an MDMA-dosage regimen sufficient to produce a large and long-lasting decrease in 5-HT was not sufficient to induce an astrocyte reaction characteristic of neural injury. Finally, at an even higher total dosage of MDMA, we were able to see an increase in GFAP (figure 6). Twice-daily administrations of 75 to 150 mg/kg of MDMA for 2 days resulted in a dose-dependent increase in the levels of GFAP in cortex and striatum at 2 days postdosing. Surprisingly, this extremely high-dose regimen resulted in a U-shaped dose-response curve with respect to levels of 5-HT; the highest total dosage of MDMA caused the least decrease in 5-HT (figure 6). Thus, evidence for MDMA-induced neural damage, as revealed by an increase in GFAP, was not necessarily linked to the propensity of the compound to cause decreases in levels of 5-HT.

In comparison with the data obtained for the dopaminergic neurotoxicants, MPTP, methamphetamine, and 6-OH dopamine, the data obtained from our studies of the putative serotonergic neurotoxicant MDMA were more difficult to interpret. With the dopaminergic neurotoxicants, increments in the concentration of GFAP in conjunction with decrements in TH (but not dopamine) could be used to estimate damage to nigrostriatal neurons, the putative targets of the neurotoxic effects of these compounds. Similarly, it would have been useful to determine whether MDMA-induced increases in GFAP were associated with decrements in the amount of tryptophan hydroxylase as well as the observed decrements in 5-HT to obtain an estimate of damage to serotonergic neurons. Our attempts to quantify tryptophan hydroxylase have not been successful; therefore, we do not know whether the increase in GFAP that accompanies the high-dose regimen of MDMA is associated with loss of serotonergic terminals or axons. However, because the 5-HT uptake inhibitor, fluoxetine, causes a partial blockade of 5-HT depletions as well as the increase in GFAP, it is likely that at least a portion of the GFAP increase emanates from an astrocyte response to damaged serotonergic neurons (O'Callaghan et al. 1991).

MPTP and 6-OH dopamine were used as positive controls in our examination of the potential for methamphetamine to induce a GFAP increase in the neostriatum of the mouse and rat, respectively. These controls were incorporated to ensure that assays of GFAP would reveal damage to dopaminergic fibers in the striatum. To achieve a similar level of confidence in the results of our MDMA studies, the serotonergic neurotoxicant 5,7-DHT was used as a positive control for damage to serotonergic neurons of the rat. Following pretreatment with desmethylimiprimine to protect catecholaminergic neurons, we gave bilateral intraventricular injections of 5,7-DHT (125 µg on each side). At 7 days after dosing, this regimen resulted in an increase (25 to 60 percent) in the concentration of GFAP in all brain areas surveyed (figure 7). 5-HT levels in these same brain regions (prepared from the same animals)

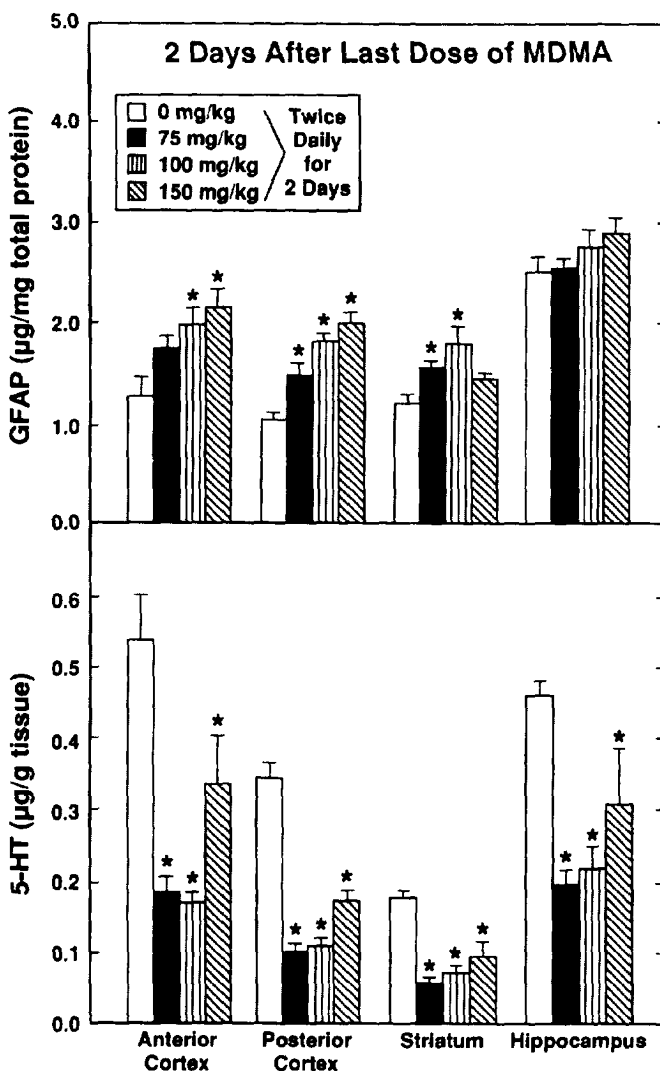

FIGURE 6. *Effects of MDMA (75 to 150 mg/kg, twice daily for 2 days) on the concentration of GFAP and 5-HT in cortex, striatum, and hippocampus of the Long-Evans rat. Rats were killed 2 days after the last dose of MDMA. GFAP and 5-HT data were generated from samples prepared from the same rats. GFAP and 5-HT were determined as described in the legends to figure 1 and figure 5, respectively.*

* Significantly different from control, $p<0.05$

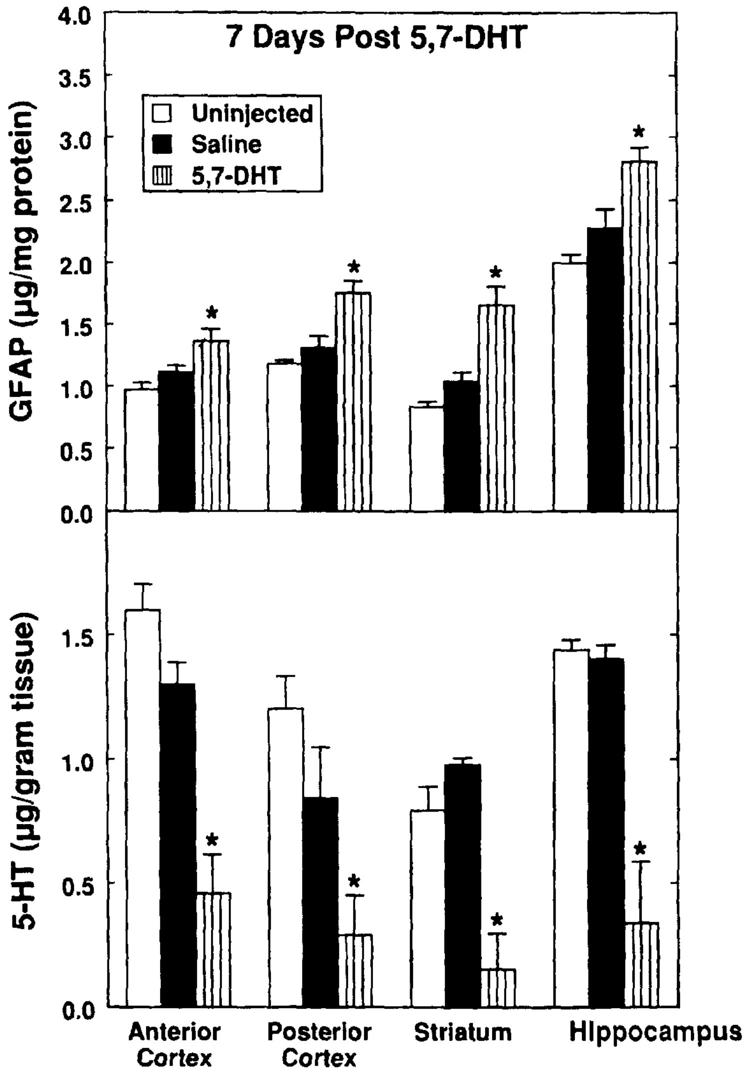

FIGURE 7. *Effects of 5,7-DHT (125 µg/side, bilateral intraventricular injections) on the concentration of GFAP and 5-HT in cortex, striatum, and hippocampus of the Long-Evans rat. Rats were killed 7 days after dosing. GFAP and 5-HT data were generated from samples prepared from the same rats. GFAP was determined by the method of O'Callaghan (1991*b*). 5-HT was determined by HPLC with electrochemical detection.*

* Significantly different from uninjected control, $p<0.05$

were reduced by 60 to 80 percent. The concentration of GFAP also was increased in most brain areas 3 days after administration of 5,7-DHT (data not shown). In general, the magnitude of the GFAP increase observed at 3 days postdosing was smaller than that observed at 1 week. Moreover, the decrements in 5-HT also were smaller at the 3-day time point in comparison to those observed at 1 week (data not shown). These data indicate that the neurodegenerative effects of the prototype serotonergic neurotoxicant 5,7-DHT result in an astrogliotic reaction as revealed by an increase in GFAP. These findings are consistent with previous observations of an increase in GFAP concentration and immunoreactivity following intrahypothalamic administration of 5,7-DHT (Frankfurt et al. 1991). The effects of 5,7-DHT on GFAP stand in contrast to the absence of any effect on GFAP with an MDMA dosage regimen that resulted in 5-HT decrements equivalent to or greater than those seen with 5,7-DHT (see figure 5). Thus, the fact that MDMA-dosing regimens that cause long-lasting decreases in 5-HT do not result in astrogliosis (figure 5) suggests that such regimens may lead to down-regulation of serotonin biosynthesis without causing nerve terminal or axonal degeneration.

UNRESOLVED ISSUES AND DATA GAPS

Based on the results of studies of a large variety of prototype neurotoxicants, the authors feel confident that assays of GFAP represent a sensitive means for detecting and quantifying neural damage. Silver staining will be revived **as** another approach for assessing toxicant-induced damage to the nervous system (Switzer 1991; Beltramino et al., this volume; Jensen et al., this volume). Each of these methods has its advantages, The GFAP-based approach is simple and quantitative, but it provides only an indirect indication of neural damage. Reduced silver methods, although more time consuming and qualitative, provide a means for the direct identification of the damaged pathways. Where GFAP assays have been combined with reduced silver methods to assess neural damage, results obtained with both techniques have proven to be similar in a number of respects (O'Callaghan and Jensen 1992). The similar sensitivity of the two procedures may be the most important shared feature for interpreting the potential neurotoxic effects of substituted amphetamines. High dosages of substituted amphetamines were required to induce an argyrophilic reaction in the affected regions of the rat brain (Commins and Seiden 1986; Commins et al. 1987; Ricaurte et al. 1984*b;* Jensen et al., this volume). Likewise, high dosages of substituted amphetamines were required to cause an increase in GFAP in the damaged brain areas of this species (figures 3 and 6). Clearly, this "high-dose" requirement does not indicate that the two approaches are not sensitive. A low dosage of MPTP in the mouse results in a large increase in striatal GFAP and an accompanying argyrophilic reaction in the nigrostriatal pathway (O'Callaghan and Jensen 1992). Similarly, a relatively modest methamphetamine-dosage regimen in the mouse also causes a marked increase in GFAP (figure 2), an effect that we predict will be accompanied by an argyrophilic reaction.

What then are we to make of our data for the effects of amphetamines in the rat? The simple conclusion is that high dosages of these compounds are required to cause neural damage in this species. Long-lasting effects of substituted amphetamines on a number of constituents of serotonergic and dopaminergic neurons have been described over the years (Gibb et al. 1990). However, one implication of these data is that these effects, when observed in the rat, may not reflect amphetamine-induced damage to serotonergic or dopaminergic pathways. These arguments are not meant to imply that most amphetamine derivatives are not detrimental to dopaminergic and serotonergic function. The long-lasting amphetamine-induced deficits in the biochemical machinery associated with these transmitter systems are suggestive, minimally, of a long-term deficit in function. Such effects can be considered adverse or "neurotoxic" to the affected pathways. Our findings emphasize the need to establish the dosage requirements for such effects in relation to the dosage requirements needed to cause neural damage. Given the differences between mouse and rat responses to methamphetamine, the cross-species generality of these dosage requirements also needs to be established for other substituted amphetamines.

Finally, we note that both astrogliosis and argyrophilia appear to represent common and, apparently, equally sensitive responses to all types of neurotoxic exposures, This observation is suggestive of the existence of a common set of "damage" signals responsible for initiating these general neurotoxic responses. No such signals have as yet been identified (O'Callaghan 1991*a*, 1991*b*; Norton et al. 1992). Elucidation of the mediators responsible for induction of astrogliosis and argyrophilia may provide the earliest and most sensitive markers for the detection and characterization of neural damage.

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DISCUSSION

Due to technical difficulties, there is no transcription available of the discussion that followed Dr. O'Callaghan's presentation.

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Pharmacokinetic Approaches to the Study of Drug Action and Toxicity

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INTRODUCTION

Pharmacokinetics refers to the events that occur following drug administration to alter the availability of a drug by elimination or metabolism. These processes contribute to the effects of a drug not only by removal from the body but, in some instances, by forming pharmacologically or toxicologically active compounds. It is therefore critical to understand the pharmacokinetics of drugs when studying their effects in vivo and in cellular systems. Because pharmacokinetics is based on measurements of the concentration of drugs and metabolites in plasma and tissue, it requires bioanalytical chemical techniques. The concentrations involved in the studies are usually in the micromolar range, and when pharmacokinetic experiments are performed, the volumes involved are small, commonly 0.1 to 0.2 mL. In the authors' studies, we have found it advantageous to perform experiments at the in vivo and in vitro levels to direct subsequent experiments or interpret results from others. The in vitro incubation mixtures usually contain cellular components, whereas the in vivo samples are plasma or tissue homogenates so that the analytes are in complex matrices. As a result, the analytical procedures require high sensitivity and specificity. This chapter describes applications of these procedures to studies of the pharmacology and toxicology of methylenedioxymethamphetamine (MDMA)- and methylenedioxyamphetamine (MDA)-related compounds.

PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES OF MDMA

MDMA is a substituted amphetamine with actions on the 5-hydroxytryptamine (5-HT) and the dopamine (DA) systems (Schmidt 1987; Stone et al. 1988; Johnson et al. 1988). The compound causes an initial release of DA as well as 5-HT but under appropriate regimens also causes a long-term depletion of 5-HT and reduction in tryptophan hydroxylase activity (Stone et al. 1989). In addition to these chemical changes, there are morphological changes consistent with a loss of presynaptic terminals in different areas of the brain (Ricuarte et al. 1985;

Wilson et al. 1989). Early studies focused on MDMA's temporal and metabolic disposition in the whole animal as it related to the pharmacodynamics of MDMA. Because MDMA is a lipid-soluble compound of moderate volatility, it was analyzable by gas chromatography (GC) techniques. The more polar catechol metabolites required liquid chromatography (LC). Therefore, the analyses utilized GC/mass spectrometry (MS) and high-performance LC/ electrochemical detection (HPLC-ECD). MS is highly sensitive, and when it was coupled to a capillary gas chromatograph, high specificity also was achieved. Similarly, ECD, when coupled to a liquid chromatograph, also was able to achieve high sensitivity and specificity for electroactive compounds. The electrochemical detection was especially sensitive for easily oxidized substrates such as the catechol function.

GC/MS With Selectedion Monitoring

A mass spectrometer is more commonly used to collect data resulting from the fragmentation of the analyte in an electron beam. A series of charged fragments is generated, and the mass spectrometer identifies the charge-to-mass ratio of each fragment and determines its relative abundance. The fragmentation pattern is characteristic of the analyte and may be used to identify or partially identify its chemical structure. In the selected ion monitoring mode, the mass spectrometer is focused on two fragments, one characteristic of the analyte and the other for the internal standard, which is frequently an isotopic variant of the analyte (Jenden and Cho 1979). Because the instrument is focused on only two masses instead of scanning a range, the limits of detectability are much lower. In the assay for MDMA, a fragment with mass/ charge (m/z) 154 was used for the analyte and a fragment of m/z 164 for the internal standard (figure 1). The peak at m/z 154 is caused by ß-cleavage of the trifluoroacetyl derivative and is the base peak or the most abundant fragment in the spectrum. The internal standard was a deuterium-substituted variant that contains two deuterium atoms in the ethyl side chain, On fragmentation, this variant has one peak at m/z 155 and a second, less intense peak (50 percent) at m/z 164. Although the m/z 155 peak would be more sensitive, the nondeuterium-enriched (d0) analyte MDMA also has a small (5 percent) quantity of fragment at this mass as well. To avoid overlap, the signal at m/z 164 with less than 1 percent overlap was chosen.

Synthesis of Internal Standards

The internal standard was prepared by the route indicated in figure 1, utilizing conventional synthetic procedures with lithium aluminum deuteride instead of the hydride (Lindeke and Cho 1972).

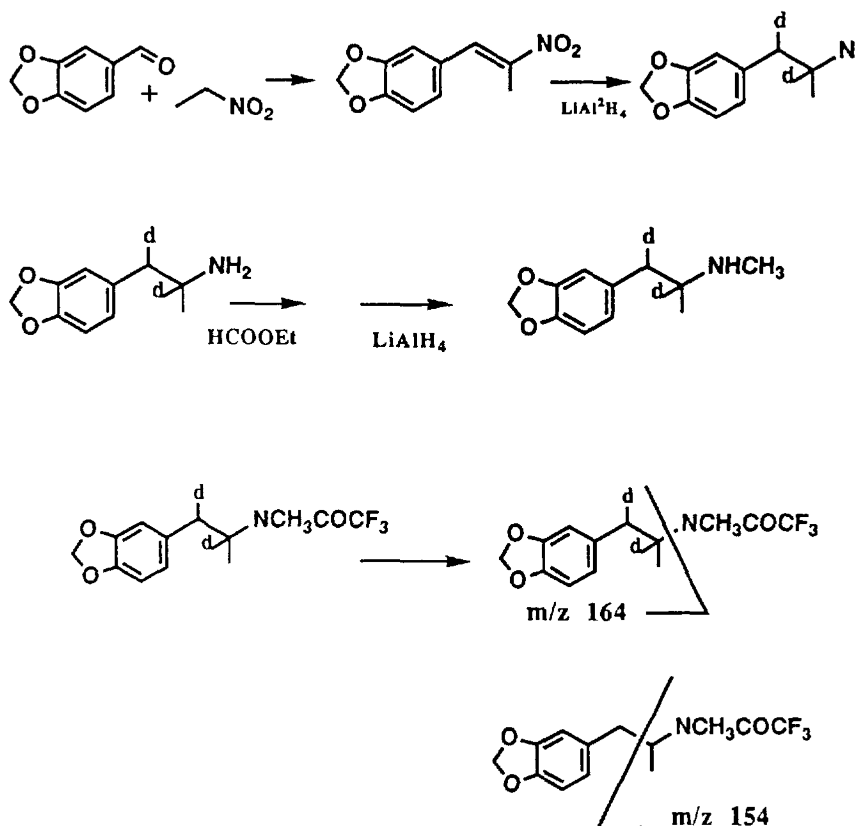

FIGURE 1. *The synthesis of deuterium-substituted MDMA and its conversion to the trifluoroacetyl derivative. The fragmentation resulting in the ions used for selected ion monitoring is shown.*

Analytical Procedure

In the analysis, the internal standard was added to the sample; and the analyte, MDMA, and the internal standard were extracted into an organic solvent. The extract was concentrated by evaporation of solvent; the residue was treated with trifluoroacetic anhydride and injected into the GC/MS. The mass spectrometer was focused on the preset masses, and the appropriate gas chromatographic peak was analyzed for ratios of signal from the analyte and internal standard. Quantitation was achieved with a standard curve generated from mixtures of fixed concentrations of internal standard and varying concentrations of analyte. Results of the initial studies determining the pharmacokinetics of MDMA after equimolar intravenous doses of (+) and (-) MDMA are shown in figure 2.

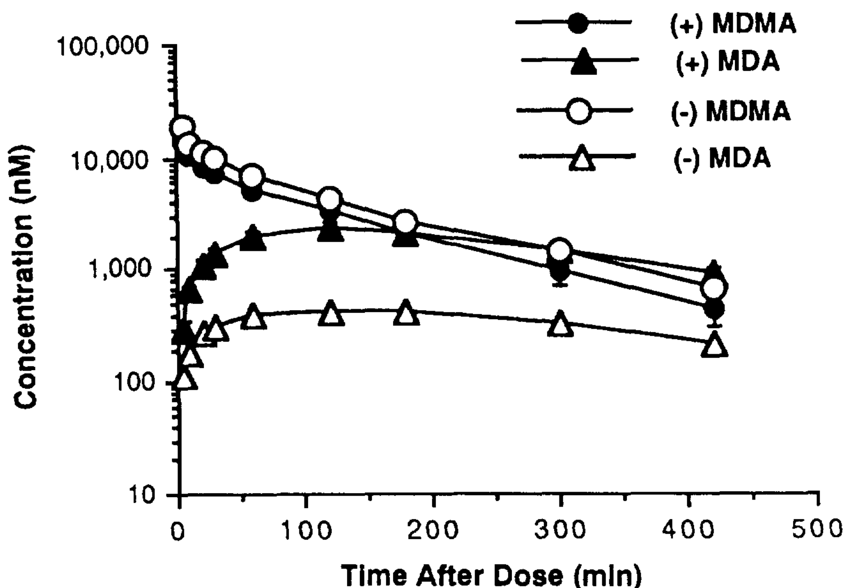

FIGURE 2. *Time course of plasma MDMA and MDA after intravenous administration of (+) and (-) MDMA (10 mg/kg)*

In Vivo Pharmacokinetics

The MDMA was administered to groups of at least five rats that had been implanted with Vascular Access Ports (Model SLA, Norfolk Medical Products, Skokie, IL) (Cho et al. 1990). These ports permit drawing of blood from unrestrained and conscious animals. Volumes of 0.5 mL of blood were collected, and the plasma (0.2 mL) was separated and analyzed by the procedures described above. The times chosen for sample collection were based on preliminary experiments and the time course of plasma decay. The object was to have data points that follow drug concentration changes with time so that parameters for the function describing the plasma time curve could be estimated. The function could then be used to (1) estimate the concentrations at time points not analyzed, (2) obtain rate constants for intercompartment transfer, and (3) estimate the area under the plasma time curve. (Intercompartment transfer refers to processes such as absorption from the site of administration, distribution throughout the body compartments, and elimination, either by excretion or by metabolism.) The data were fitted to the pharmacokinetic model shown in figure 3, an open two-compartment model, reflecting the movement of drug from the central to the peripheral compartment (k_{12}, k_{21}), through metabolism to MDA (k_1, k_4) and other pathways of elimination (k_3) The fitting was

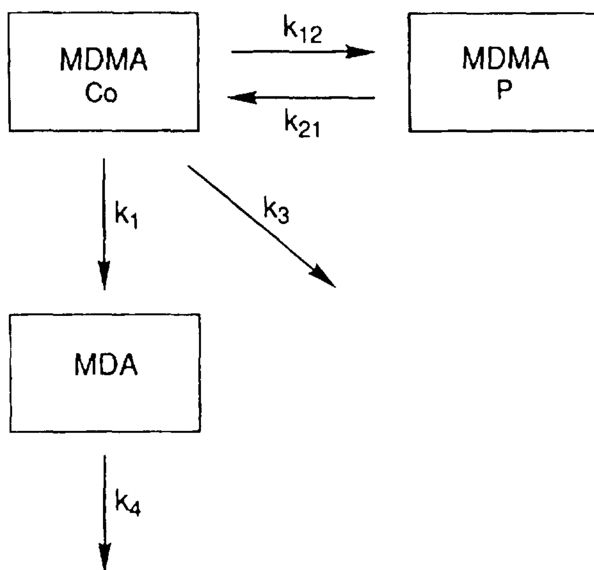

FIGURE 3. *Compartment model used to analyze the pharmacokinetic data of figure 2*

accomplished by nonlinear regression procedures (Motulsky and Ransnas 1987) using Dixon's BMDP software program on a microcomputer (Dixon 1985). In this regression procedure, the data are interactively fitted to an exponential function that is the solution to the differential equation describing the plasma decay curve. The function can be set up on a spreadsheet and the concentrations at any time calculated by substituting the desired time. This method of data analysis provides estimates of rate constants (table 1) together with estimates of the errors in their values, The significance of the constants can be evaluated using the F value (Motulsky and Ransnas 1987). The values of the individual rate constants or microparameters are useful in evaluating the relative importance of different metabolic processes, For example, the ratio of k_1, the rate constant for MDA formation, to k_3, the rate constant representing all other pathways of elimination, is about 10. This means that MDA formation represents only a small proportion of the total drug elimination process (-10 percent), The relative values of k_3 for the (+) and (-) isomers indicate a substantial enantiospecificity in MDA formation. The effect of pharmacological manipulation of the rate constants is shown in table 1 also. Phenobarbital pretreatment, which commonly induces cytochrome P450 isozymes, did not appear to alter the rates of demethylation but had a significant effect on k_3, the alternate pathways of elimination. At the

TABLE 1. *Microscopic rate constants-MDMA*

Treatment	k_1 (min^{-1})	k_3 (min^{-1})	k_3 (min^{-1})
(+)MDMA, control	.0025	.0240*	.0058
(-)MDMA, control	.0006	.0190	.0062
(+)MDMA, phenobarbital	.0026	.0354†	.00103
(-)MDMA, phenobarbital	.0011	.0378	.0093
(+)MDMA, SKF 525A	.0022	.0202*	.0058
(-)MDMA, SKF 525A	.0004	-.0207†	-.0059

* p<.01 vs. (-) control
†p<.01 vs. (+) control

doses used, the cytochrome P450 inhibitor SKF 525A appeared to decrease the values of k_3 for the (+) but not the (-) isomer.

The results show that the outcome of pretreatment is not always predictable. Thus, phenobarbital induction did not increase levels of MDA as might be expected. Instead of increasing the rate of MDA formation, induction increased the rate of MDA elimination.

In Vitro Metabolism

The pharmacokinetic data showed that MDA formation is a relatively minor pathway in the overall elimination pathways of MDMA (Hiramatsu et al. 1990). To evaluate other pathways, in vitro experiments were conducted to obtain quantitative data of regiospecificity (Kumagai et al. 1991*a*). These studies required the analysis of highly polar catecholamines and their derivatives (figure 4). To study the overall metabolism, the aforementioned GC/MS assay was used along with an additional HPLC/ECD procedure (Hiramatsu et al. 1990). The results, shown in table 2, reveal that the catecholamine dihydroxy methamphetamine (DHMA) is the dominant metabolite, occurring at levels 10 times greater than the others (Kumagai et al. 1991*a*). In spite of the extensive demethylenation in vitro, there was very little if any DHMA present in any of the plasma samples assayed, including the early time points. An explanation for this discrepancy was found in direct pharmacokinetic evaluation of α-methyldopamine (α-MeDA) because the N-methyl analog was not available in sufficient quantity to study in this manner. The pharmacokinetic parameters obtained after equimolar, intravenous doses of a-MeDA and MDA are shown in table 3. The value of k_2 for α-MeDA, the rate constant for elimination, was found to be 10 times greater

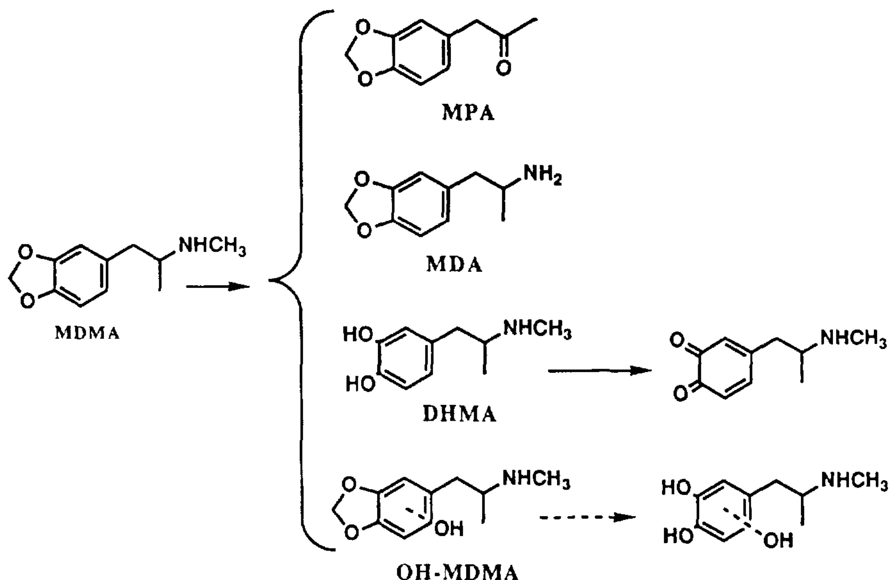

FIGURE 4. *Metabolic pathways of MDMA*

TABLE 2. *Metabolism of MDMA by liver microsomes*

Species	DHMA	MDA	MPA	OHMDMA
Rat (+)*	5.37	0.21	0.12	.025†
Rat (-)*	3.35	0.31	0.05	.010†

* Nmols of product formed in 5-minute incubation of 30 nmols MDMA
†approximate

KEYS: MPA=methylenedioxy phenylacetone;
OHMDMA=hydroxymethylenedioxy methamphetamine

than that for MDA. The rate constant for α-MeDA is equivalent to a half-life of about 2 minutes, compared with 22 minutes for MDA. Thus, even with a large rate of formation, the high rate of elimination precludes accumulation of significant levels of the catechol in plasma.

In Vitro Toxicity

Methods also are being developed to study the chemical mechanisms of neurotoxicity utilizing a neuroblastoma glioma clonal cell line, NG 108-15

TABLE 3. *Microscopic* rate *constants-MDA and* α-*MeDA*

Compound	Vd (1/kg)	k_{21}	k_2 (min^{-1})	k_{12}	$t_{1/2}$ (min)
α-MeDA	0.16	.059	.360	.048	1.92
MDA	0.76	.079	.032	.219	21.65

NOTE: The open, two-compartment parameters are: Vd is the volume of distribution of the central compartment; k_2 is the rate constant for elimination: k_{12}, k_{21} are rate constants for fluxes to and from the central compartment. The half-life, $t_{1/2}$, was calculated from k_2.

(Patel et al. 1991). This cell line has been described by Furuya and associates (1985) as having features associated with the 5-HT nerve terminal such as the ability to store, release, and accumulate 5-HT. The intention is to use such a model to test metabolites of MDMA and other neurotoxins. These cells contain high levels of glutathione (GSH), a peptide that protects cells against insult by electrophiles, free radicals, and metals by undergoing addition, oxidation, and complexing reactions, respectively (Reed 1985). The high levels of GSH in this cell line and the availability of depleting agents permits the role of this agent in protection against toxins to be examined experimentally.

The proposed metabolism of MDMA to potentially toxic metabolites is shown in figure 4. In this sequence, MDMA can undergo demethylenation to the catechol DHMA (Kumagai et al. 1991 *a)* or hydroxylation to the Ar-hydroxy compound. In studies reported elsewhere (Kumagai et al. 1992), a ring-hydroxylated metabolite of MDMA has been characterized, but the location of the hydroxyl group has not been established. DHMA can be generated directly by a cytochrome P450-dependent reaction or by a hydroxyl radical-dependent oxidation (Kumagai et al. 1991*b*). Catechols are readily autoxidized to the o-quinone, and this reaction, together with a rapid addition of GSH, has been reported (Hiramatsu et al. 1990). However, in low-GSH conditions, the o-quinone can react with nucleophilic centers in biological molecules and cause a biochemical lesion (Graham et al. 1978; Slaughter and Hanzlik 1991). Alternatively, the proposed Ar-hydroxy metabolite can be generated, which also can undergo demethylenation to form the highly reactive trihydroxy derivative. The position of ring hydroxylation of MDMA has since been identified as the 6-position based on comparison of HPLC retention times and GUMS data with authentic material. These functions can cause cellular toxicity by reduction of oxygen to superoxide catalytically, acting as an electron transfer agent between reduced nicotinamide adenine dinucleofide phosphate and oxygen (Graham et al. 1978). Superoxide rapidly

disproportionates to oxygen and hydrogen peroxide, and its continuous formation by the trihydroxy compound can result in hydroxyl radical formation through the Haber Weiss reaction. Hydroxyl radical is highly reactive and thought to be the basis for toxicity of a variety of oxidizing agents (Cohen 1984). GSH would protect the cells against the electrophile- and radical-based insults shown above so that its depletion should alter the toxicity of agents whose action proceeded by this mechanism.

In initial studies, the interaction of the metabolite of MDA, α-MeDA, was compared with DA for its toxic actions on the cell line (Patel et al. 1991). Because both compounds contain the catechol moiety, toxic actions based on o-quinone formation should be very similar, reflective of the availability of the quinone. Toxicity was measured as cellular destruction and required fairly high concentrations *of* the compounds. The results are shown in table 4 as EC_{50} values, the concentration necessary to kill 50 percent of the cells. The value for DA was almost four times higher than that for α-MeDA. The total quantities of catechol accumulated, on the other hand, were much greater after DA (Patel 1990). When the GSH in the cells was depleted by the synthesis inhibitor, buthionine sulfoximine (BSO), the toxicity of the two compounds increased, but the increase was much greater for α-MeDA. Thus, the results of the study showed that, although DA is accumulated by the cells much more extensively, it is much less toxic than its α-methyl analog. Furthermore, its toxicity does not increase in the same proportion on GSH depletion.

One explanation for this discrepancy is in the intracellular disposition of the two compounds (Patel et al. 1991). DA is a neurotransmitter, and its removal from cytoplasm by storage and monoamine oxidase-initiated metabolism resulted in low availability. In support of this notion, DA accumulation was found to

TABLE *4. Interaction of catecholamines and NG 108-15 cells*

	EC_{50}* (mM)	
Compound	Control	BSO Treated†
DA	24.5	10.5
α-MeDA	6.8	0.6

* Concentration required to kill 50 percent of the cells after a 2-hour exposure
† Cells treated with buthionine sulfoximine (BSO, 2 mM) for 24 hours prior to drug exposure (Patel et al. 1991)

decrease substantially in reserpine pretreated cells, whereas the accumulation of α-MeDA was not affected (Patel 1990). The metabolism of DA by monoamine oxidase to dihydroxyphenylacetic acid and homovanillic acid is not available to α-MeDA, although the latter compound may be converted to its 3-methyl derivative by catechol o-methyltransferase. The major transformation of α-MeDA appears to be the GSH adduct, originally found in incubates of liver preparations (Hiramatsu et al. 1990). Although the GSH adducts are formed from both DA and α-MeDA, the early levels of the latter are much higher. The intracellular concentration of GSH is in the millimolar range and far exceeds the concentration of the catecholamines so that the rate of formation of GSH adduct is dependent on catecholamine availability. Therefore, the concentration of α-MeDA and its quinone oxidation product must be higher and available for binding to cellular nucleophiles. The GSH adducts appear to be excreted by the cell, and analysis of the culture medium after incubation has shown that there are micromolar levels of the GSH adduct of α-MeDA present, whereas, similar to DA, GSH concentration was below detectable limits. Thus, α-MeDA is extensively converted to the GSH adduct of the o-quinone, whereas DA appears to have limited conversion. If the concentration of this adduct reflects that of the toxic o-quinone, the greater toxicity of α-MeDA can be accounted for by its greater availability.

ACKNOWLEDGMENT

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DISCUSSION

Due to technical difficulties, there is no transcription available of the discussion that followed Dr. Cho's presentation.

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Mechanisms Mediating Biogenic Amine Deficits Induced by Amphetamine and Its Congeners

James W. Gibb, Michel Johnson, Donna M. Stone, and Glen R. Hanson

INTRODUCTION

In other chapters of this monograph, evidence is presented that the neurotoxicity of drugs of abuse can be assessed by monitoring behavioral, neurophysiologic, and neuroanatomic parameters. In this chapter, evidence is cited that neurotoxicity also can be assessed by determining the effects of drugs of abuse on neurochemical elements. Because Gibb and colleagues at the University of Utah have investigated primarily methamphetamine (METH) and its congeners over the past two decades, this group of abused agents is used as an example. In monitoring neurochemical parameters, one can measure neurotransmitter concentrations ex vivo or by in vivo dialysis. Enzyme activity, either V_{max} and/or K_m, can be determined. Loss of uptake sites (Battaglia et al. 1987) or alteration of binding sites also can be assessed.

In 1971, Koda and Gibb (1971, 1973) attempted to simulate in rats the conditions that exist in METH abusers who characteristically inject the drug intravenously at frequent intervals over a period of 2 to 3 days. METH (10 to 15 mg/kg, subcutaneously [SC]) was administered every 6 hours for 30 hours, and the effect on tyrosine hydroxylase (TH) activity and on the concentrations of dopamine (DA) and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in the neostriatum was determined. Following drug treatment, these parameters were decreased to approximately 60 percent of normal. Because it was known that amphetamines release catecholamines, it was not surprising that the concentrations of DA and its metabolites were depressed, but an explanation for the decrease in TH activity was not immediately apparent. Induction of TH in the adrenal gland, superior cervical ganglion, and selected areas of the brain had been reported in stressed animals (Zigmond et al. 1980; Masserano et al. 1981) but a decline in the activity of this enzyme was rather perplexing; it was acknowledged that 6-hydroxydopamine (6-OHDA), a potent neurotoxin, also decreased TH activity.

After a comparable dosing schedule, METH had similar effects on the serotonergic system (Hotchkiss et al. 1979; Hotchkiss and Gibb 1980). Tryptophan hydroxylase (TPH) activity was markedly decreased after a single dose of METH; a decrease in concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) was also observed. A major difference in the response of the 5-HT and DA systems was the more rapid onset and more profound response observed in the 5-HT system compared with the DA system. The question then arose as to whether these large, repeated doses of METH were neurotoxic to all neurotransmitter systems or to selected neurons. The effect of METH on the cholinergic system was assessed by measuring choline acetyltransferase activity in the neostriatum, nucleus accumbens, and olfactory tubercle after METH; because this enzyme, which is responsible for acetylcholine synthesis, was not altered, it was concluded that cholinergic systems remained intact after METH (Hotchkiss et al. 1979). Using a similar strategy, glutamic acid dehydrogenase was measured to determine whether the γ-aminobutyric acid (GABA) system was affected; the activity of this enzyme was unaffected. It appears, then, that the neurotoxic effect of large doses of METH is limited to the dopaminergic and serotonergic systems.

Ricaurte and colleagues (1985) reported that an analog of amphetamine, 3,4-methylenedioxyamphetamine (MDA), decreased concentrations of neostriatal 5-HT and 5-HIAA but had no effect on DA or norepinephrine content. Morphological changes, as demonstrated by the Fink-Heimer technique, also were observed: moreover, 5-HT uptake sites were compromised. Stone and coworkers (1986) examined the effects of MDA and 3,4-methylenedioxymethamphetamine (MDMA) on the activity of both TPH and TH. Whereas METH characteristically decreases both TH and TPH activity, MDA or MDMA depressed only TPH activity. The more selective effect of MDMA or MDA on the serotonergic system is reminiscent of the response to p-chloroamphetamine.

A compound cannot be assumed to be neurotoxic if the neurochemical changes are transient: however, when neurochemical alterations persist, neurotoxicity is suspected. Bakhit and colleagues (1981) and Stone and colleagues (1987) administered multiple doses of METH or MDMA to rats, stopped the drug, and waited for varying periods of time after the dosing was discontinued to measure the neurochemical response. When five administrations of MDMA or METH (10 mg/kg) were given, TPH activity and concentrations of 5-HT and 5-HIAA were still depressed at 110 days. Because MDMA affects the serotonergic system selectively, comparable results were obtained for the dopaminergic system only when METH was administered. These data, coupled with the morphological evidence from other laboratories (Ricaurte et al. 1984; Commins et al. 1987; O'Hearn et al. 1988), are highly suggestive that large doses of METH are neurotoxic to dopaminergic neurons, whereas MDMA is selectively neurotoxic to the serotonergic system.

DOPAMINE IS ESSENTIAL FOR THE NEUROTOXIC RESPONSE

The mechanism by which the neurotoxic effect of the amphetamine analogs occurs has been under consideration from the very beginning of Gibb and colleagues' studies. Buening and Gibb (1974) reported that the DA antagonists, chlorpromazine and haloperidol, prevent the usual response to METH. In subsequent studies, Sonsalla and coworkers (1986*a*) observed that either D_1 or D_2 antagonists block the effects on the DA system; the effects of METH on the 5-HT system were blocked only by D_1 antagonists.

Further evidence that DA is essential for the METH-induced neurotoxicity was then obtained (Gibb and Kogan 1979; Hotchkiss and Gibb 1960; Schmidt et al. 1965). DA synthesis was blocked at the rate-limiting step by inhibiting TH by administering α-methyl-p-tyrosine (MT). The METH-induced alterations in both the dopaminergic and the serotonergic systems were prevented by concurrent administration of MT. When synthesis of DA was reinstated by administering L-dopa concomitantly with the METH and MT, the neurochemical deficits returned.

Recently, it was found that when the nigrostriatal dopaminergic projections are destroyed by prior injection of 6-OHDA into the substantia nigra, METH (Johnson et al. 1967; Sonsalla et al. 1966*b*) or MDMA (Stone et al. 1968) does not cause the characteristic decrease in striatal TPH activity. However, METH or MDMA still decreases TPH activity in the cerebral cortex or hippocampus of 6-OHDA-pretreated animals. Thus, dopaminergic innervation is critical for the METH or MDMA to cause the neurotoxic response.

Catecholamines were then depleted by prior administration of reserpine. In animals so depleted, MDMA was ineffective in causing its usual deficit in TPH activity (Stone et al. 1988). This observation provides further evidence that DA is essential for the neurotoxicity observed after MDMA or METH. Additional evidence for the necessity of DA in the neurotoxic process was recently provided by Chris Schmidt (personal communication, May 1991), who demonstrated that L-dopa enhanced the deficits in rat brain 5-HT content 1 week after administering an otherwise ineffective single dose of MDMA or METH.

IS GLUTAMATE INVOLVED IN THE NEUROTOXICITY?

Sonsalla and colleagues (1989) recently reported that MK-801, an N-methyl-D-aspartate (NMDA) antagonist, protects against the METH-induced deficits in the dopaminergic and serotonergic systems, which suggests that glutamate is associated with the METH-induced neurochemical events that lead to toxicity. Johnson and coworkers (1989) confirmed these observations but found that MK-801 does not protect against the MDMA-induced neurotoxicity. Glutamate appears to play a role in the METH-induced neurotoxicity but not in the MDMA-induced deficits.

ROLE OF AN OXIDATIVE PROCESS IN NEUROTOXICITY

The above data provide significant evidence that DA and/or its reactive metabolites are essential for neurotoxicity. The question remains as to the mechanisms by which DA causes the response. Stone and colleagues (1989) have evidence that an oxidative process is responsible for the serotonergic alterations associated with the neurotoxicity. It was reported earlier that TPH activity is rapidly decreased within 15 minutes after a single dose of METH (Bakhit and Gibb 1981). Schmidt and Taylor (1987) reported no change in the K_m for substrate or pterin cofactor, but a decrease in V_{max}, was observed after MDMA. TPH is activated by a variety of conditions, including exposure of the enzyme to certain phospholipids, mild proteolysis, and phosphorylating conditions (Hamon et al. 1981; Vitto and Mandell 1981; Garber and Makman 1987); under these conditions, the affinity of the enzyme is increased, and the apparent K_m for tryptophan and/or the pterin cofactor is decreased.

In contrast, a strong reducing environment (5 mM dithiothreitol [DTT] and 50 mM Fe^{2+} under a nitrogen atmosphere) increases the apparent V_{max} of TPH but does not alter the apparent K_m (Hamon et al. 1978). It has been previously reported that in vitro exposure of TPH to oxygen inactivates the enzyme (Kuhn et al. 1980). The oxygen-inactivated TPH can be reactivated by incubating the enzyme in an anaerobic milieu under reducing conditions. The similarity between the inactivation by molecular oxygen and by MDMA prompted Stone and colleagues (1989) to investigate the role of enzyme oxidation in the acute decline of TPH by MDMA.

Rats were administered a single dose (10 mg/kg) of MDMA and killed 3 hours later. Crude enzyme from the cerebral cortex (27,000 x g supernatant) from saline- or MDMA-treated rats was placed in test tubes, and DTT and Fe^{2+} were added. The preparation was incubated under nitrogen at 25°C for varying periods of time, and TPH activity was assayed by a modified $^{14}CO_2$-trapping procedure (Ichiyama et al. 1970). The time course of in vitro reactivation is depicted in figure 1. Addition of DTT and Fe^{++} to the enzyme from saline-treated rats prevented deterioration of the enzyme activity observed with incubation in water. When the reducing mixture was incubated with TPH from MDMA-treated rats, enzyme activity was restored from 40 percent of control to normal over a 24-hour incubation period. Several other sulfhydryl-reducing agents were examined for their ability to reactivate MDMA-inactivated TPH. Although all compounds slightly elevated the inactivated enzyme, only DTT and 2-mercaptothanol caused significant reactivation of MDMA-depressed enzyme during the 24-hour preincubation period.

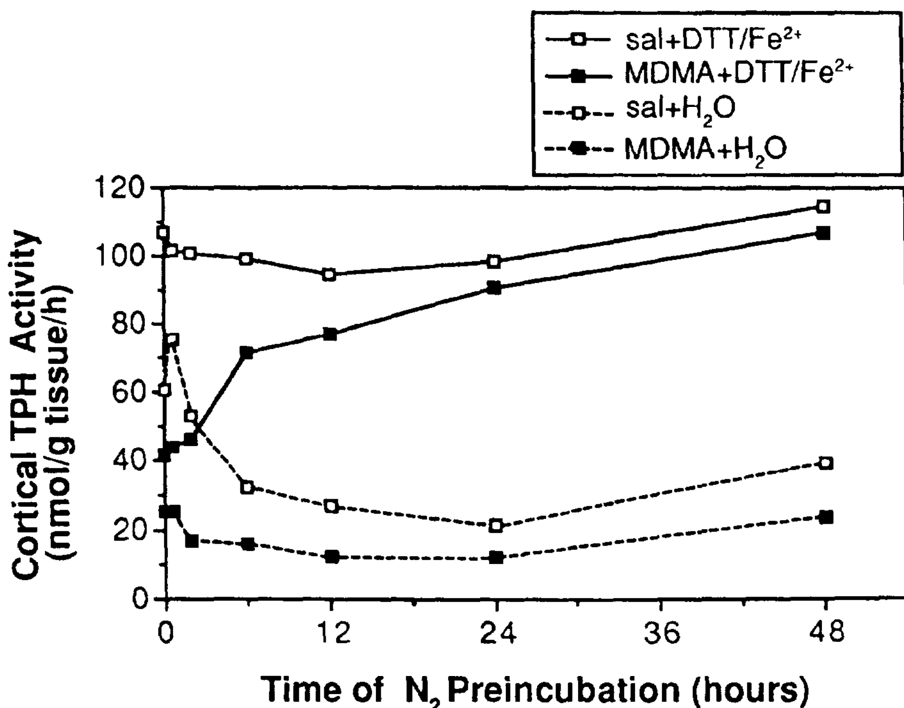

FIGURE 1. *Time course of in vitro reactivation of MDMA-inactivated cortical TPH. Enzyme solutions were prepared from rats killed 3 hours after a single SC injection of MDMA (10 mg/kg) or saline (sal). Reactivation mixture (DTT/Fe^{2+}) or water was added to enzyme preparations, which were then assayed immediately (zero time point); the remainder was subsequently preincubated under N_2 for up to 48 hours. Each point represents the mean of four determinations.*

SOURCE: Stone et al. 1989. Copyright 1989 by *Journal of Neurochemistry.*

Because MDMA decreases TPH activity by decreasing V_{max}, rather than the affinity for either the substrate or cofactor (Schmidt and Taylor 1987) the effect of reducing conditions on the kinetics of the enzyme was determined (figure 2). When cortical TPH from MDMA-treated rats was incubated under reducing conditions, enzyme reactivation essentially doubled the V_{max} of TPH, whereas the K_m for either tryptophan or the cofactor, 8-methyl-5,6,7,8-tetrahydropterin (6-MPH_4), was not significantly altered.

Insight into the time course of the toxicity induced by MDMA in vivo could likely be obtained by assessing the ability of reducing conditions to reactivate the

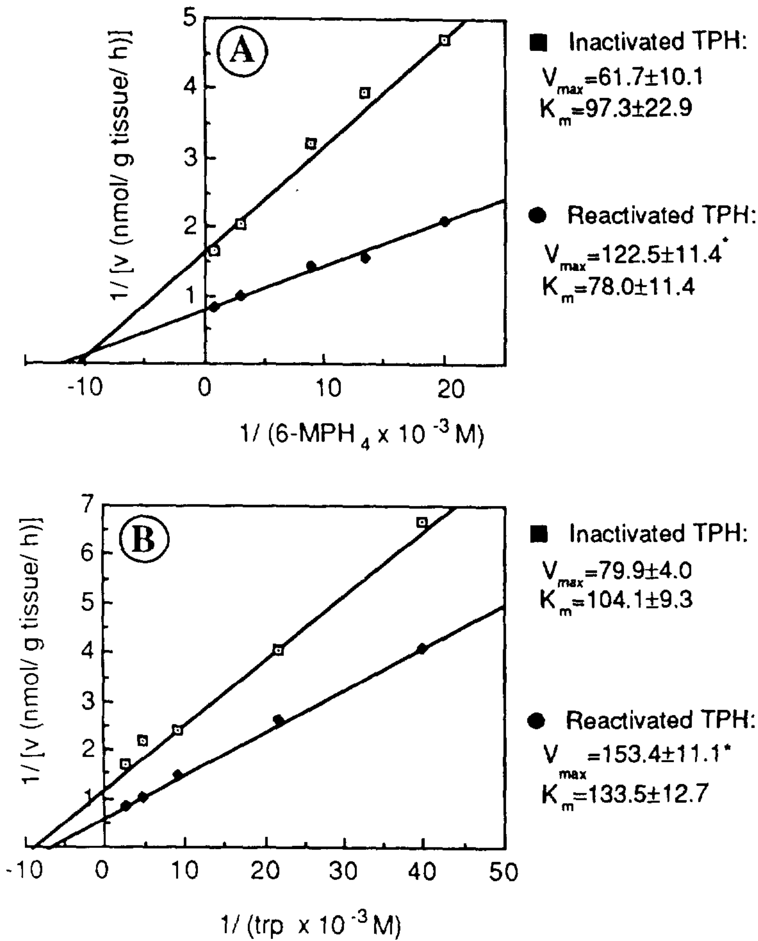

FIGURE 2. *Double reciprocal plots of inactivated and reactivated TPH activity from MDMA-treated rats, as a function of 6-MPH$_4$ (A) or tryptophan (trp) (B) concentrations. TPH solutions were prepared from rats killed 3 hours after a single dose of MDMA (10 mg/kg, SC). Following addition of reactivation mixture, inactivated and reactivated enzyme solutions were preincuba ted for 24 hours in room air at 0°C and under N_2 at 25°C, respectively. The K_m and V_{max} values are the means&EM derived from double reciprocal plots of each of three enzyme samples (each sample was prepared from the pooled cortices of five rats). The lines shown are based on the mean K_m and V_{max} values; each point represents the mean value for l/velocity at that substrate concentration.*

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enzyme at increasing times after administering the drug. If less and less enzyme is reactivated as the time of in vivo exposure to MDMA increases, this would suggest the development of irreversible drug-induced damage. Rats were killed at varying times after MDMA administration, and the enzyme was incubated under reactivating conditions (table 1). Neurotoxicity occurred rapidly following MDMA (10 mg/kg).

Within 6 hours after MDMA was administered, <50 percent of inactivated TPH could be reactivated; by 3 days, essentially all the MDMA-inactivated enzyme (>80 percent) had been irreversibly altered.

TABLE 1. *Cortical TPH activity from rats killed at various times after saline or MDMA treatment, before and after preincubation under reactivating conditions*

	TPH Before Preincubation		TPH After Preincubation		
Time	Saline	MDMA	Saline	MDMA	Percent Recovery
3 hours	98.0±5.9	54.4±10.3 (55.6±10.5)	91.5±6.8	93.1±9.0 (101.7±9.8)†	103.8
6 hours	103.±8.2	38.0±5.1 (36.9±5.0)	100.4±6.8	69.8±6.6 (69.5±6.5)†	51.7
3 days	99.6±3.1	48.6±4.0 (48.8+4.0)	91.1±4.9	51.8±4.9 (56.9+5)	15.8
1 week	80.6±2.9	50.2±2.9 (62.4±3.6)	82.9±6.0	52.6+5.0 (63.5±6.0)	2.9
30 days*	53.7±4.5	29.9±2.7 (55.7±5.0)	65.4±3.9	30.3±3.2 (46.3±4.9)	-21.2

NOTE: Rats were killed at indicated times following a single saline or MDMA (10 mg/kg) injection. Cortical TPH activity (nmol/g of tissue/hour) from each rat was assessed following the addition of reactivation mixture, both before and after a 20- to 24-hour preincubation under N_2. Results are presented as the means&EM (n=5 to 7). Numbers in parentheses indicate percentage of saline control. Percent recovery was calculated as: ([percent after preincubation - percent before preincubation]/[100 - percent before preincubation]) X 100.

*30 days after multiple MDMA doses (10 mg/kg per dose: five doses, one every 6 hours)
†p<0.05 vs. corresponding percentage before preincubation

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Because congeners of MDMA also decrease TPH activity, it was determined whether enzyme from animals injected with a single dose of METH (10 mg/kg), p-chloroamphetamine (5 mg/kg), or MDMA (10 mg/kg) could be restored by incubating under reducing conditions (figure 3). Incubation with DTT/Fe^{2+} for 24 hours reactivated the TPH from animals treated with each of the congeners. It appears that oxidative inactivation is common to the neurotoxicity of all three amphetamine congeners.

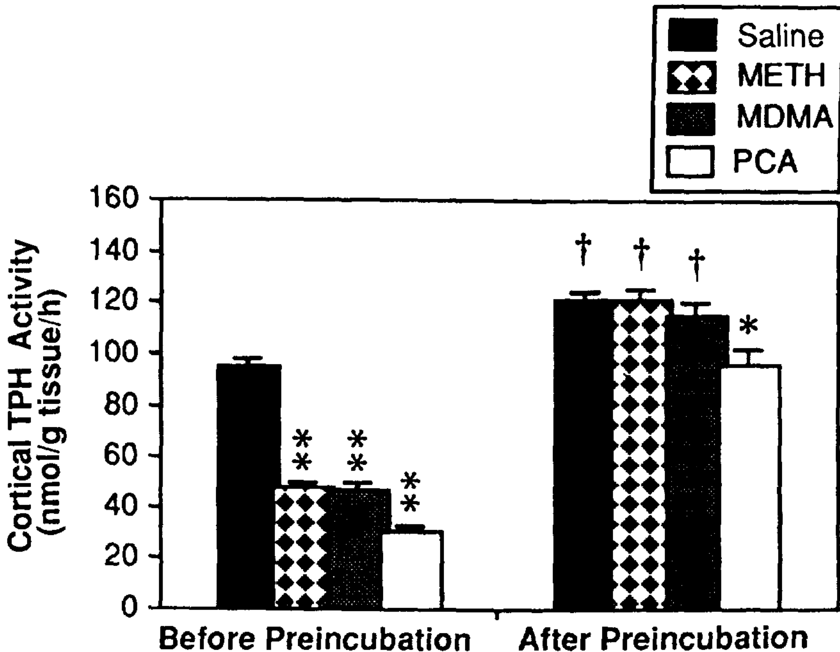

FIGURE 3. *Comparison of cortical TPH activity, before and after reactivation, from rats treated with MDMA, METH, or p-chloroamphetamine (PCA). Rats were administered a single SC injection of MDMA (10 mg/kg), METH (10 mg/kg), PCA (5 mg/kg), or saline and killed 3 hours later. Cortical TPH activity (nmol/g of tissue/hour) was assessed following the addition of reactivation mixture, both before and after a 24-hour preincubation under N_2. Results are the means&EM (n=6).*

*p<0.05

**p<0.001 vs. time-matched saline

†Denotes a significant difference (p<0.01) between percentage after preincubation and percentage before preincubation (percent=TPH activity from drug-treated rats/TPH activity from time-matched saline-treated rats] X 100)

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CONCLUSIONS

Amphetamine and its analogs cause neurochemical deficits in brain dopaminergic and serotonergic systems. Because these deficits persist for long periods after discontinuing the drug, neurotoxicity is inferred. Endogenous DA is essential for neurotoxicity to occur. TPH, inactivated by administering amphetamine congeners to rats, can be reactivated by incubating the enzyme under reducing conditions; this suggests that the serotonergic deficits are associated with oxidative reactions.

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DISCUSSION

Q: Methamphetamine, do you have a single dose?

A: Do I have a single dose? I don't have one here, but we do have it. You get more effect with amphetamine on the dopamine system than on the serotonergic system. And so, obviously with one dose, you are not going to see much effect on the dopamine system.

Q: You give high doses?

A: Yes.

Q (Seiden): I wonder if you could state whether you think that the dopamine is exerting its effects by activating other neurons or is it exerting some effects on the chemistry of the system independent of neuroactivity?

A: I don't know the answer to your question, but my speculation would be that the dopamine is auto-oxidized and that auto-oxidation results in the formation of hydroquinone or in free radicals. I think those reactive metabolites might be

responsible for causing the neurotoxicity. At this point that's my guess, and only time will tell, after the appropriate experiments have been performed.

Q (Seiden): Just before I left for this meeting, I read a paper in which they showed that 5,7-DHT could catalyze the reduction of glutathione.

A (Gibb): That should be explored.

Q (Ricaurte): Could you comment on the difference between an amphetamine and methamphetamine? The basis for the question is that if dopamine release is the key, and both amphetamine and methamphetamine are very good dopamine releasers, both would be expected to have similar neurotoxic properties. Yet there is no question that methamphetamine is much more toxic than amphetamine to serotonin systems.

A: Yes

Q (Ricaurte): Why do you think that there is such a difference?

A: I don't know. There are obviously some factors that still need some sorting out. In our hands at least, we see toxicity with both. But there is more: I agree with you. In our hands, compared to amphetamine, methamphetamine is more toxic to the serotonergic than to the dopaminergic system.

COMMENT (Cho): Let me comment on that. We have done some studies comparing methamphetamine- and amphetamine-induced release of dopamine after an intravenous dose, and it is very clear that the rate at which dopamine comes out is much greater with methamphetamine. I think that this is just a question of the rapidity with which methamphetamine gets into the brain. The volume of distribution of methamphetamine is much larger than amphetamine. I think that is because the stuff gets in the brain more quickly and it is causing release faster. So it's like comparing morphine and heroin.

Q (Cho): I want to go back to your thiol protein recovery experiments. When you did the thiol experiments, was this a whole cell preparation or was it cell-free?

A (Gibb): We homogenized and spun it down at 27,000 g. So this was 27,000 g supernatant.

Q (Cho): I guess what I am really asking is where is the enzyme that you are activating? Is it the bottom of the cell particle?

A (Gibb): Yes, I understand your question. I don't know the answer, but the 27,000 g supernatant still has cell particles.

COMMENT (Cho): Sometimes glutathione has trouble getting access to sites, and that was why I was asking that question.

COMMENT (Gibb): In other words, you are saying that glutathione can't get in.

Q (Cho): If you could get a soluble enzyme, you could go after it. The other question is why you used iron 2 with the DTT?

A (Gibb): Thank you for bringing that up. I should have mentioned it. The iron is not essential in the cortical preparation, but in the striatal preparation, it enhances the response. I don't know why. Perhaps there is enough iron bound to the enzyme that you don't need the iron. But if you get a more purified enzyme, then iron is essential.

Q (Cho): What do you think iron is doing?

A: I don't know.

COMMENT (Cho): If you have iron 2 and you continually generate it (presumably you would with the thiol reagent present), you would have ideal conditions for generating hydroxyl radical, because it is the iron 2 and oxygen and peroxide that generate this stuff. So, in a way, within that system, it sounds to me as if you were fighting the cell. That was the thing that should have bothered me.

COMMENT (Gibb): I suppose there are enough protective capabilities within the preparation that that is not occurring.

COMMENT (Cho): But then I looked at your cortical preparation, and you didn't get any better results in the absence of iron.

COMMENT (Gibb): And the only reason we included it, Art, was that in the neostriatum it does seem to improve the situation to some extent. That has been observed not only with MDMA but with oxygen as well.

Q: What do you think we should make of what looks like an increase in TPH activity as you use agents that modify the dopamine release?

A (Gibb): Yes. That is an inconsistent finding, but I think that it is fascinating; and I think that it demonstrates that there is possibly some interaction that is going on between the dopamine and the serotonergic system.

Q: Maybe it is a basal interaction?

A (Gibb): Yes. I think that has been demonstrated neurophysiologically as well as neurochemically. But what controls that interaction, I am not sure. But it is very pronounced. We can get up to 130 percent of control when we give reserpine or somehow interfere with the dopamine input to the serotonergic system.

Q: It could be determined by your basal dopamine?

A (Gibb): Yes, but I think there are those projections, those interactions that are very real.

Q (Seiden): Did you have any EDTA [ethylenediaminetetraacetic acid] present?

A: No, we didn't.

Q (Olney): Why does MK801 protect?

A (Gibb): That is a good question. Based on some other experiments that we have done, we think that there is a link between the dopamine and the glutamate systems. Glen Hanson has done a lot of experiments with the neuropeptides, which is another story and is very fascinating, and I think that it offers another way that we can get at some of these problems. But it seems that when we give NMDA, we have been aggravating the situation. It appears that way, that maybe it is a sequence.

COMMENT (Olney): So there is an NMDA receptor involved?

COMMENT (Gibb): Yes. I am sure that you are happy about that.

COMMENT (Olney): Well, I am puzzled.

COMMENT (Gibb): So are we at this point,

COMMENT (Olney): I am also happy. Makes things nice and challenging and complicated. Ordinarily, you would think of an NMDA receptor being on dendrites or cell bodies; and if it is mediating any toxicity, it would be toxic to the cell bodies and dendrites. That is the conventional arrangement. But you are dealing here with an apparent toxic effect impinging on axons and axon terminals.

COMMENT (Gibb): We are.

COMMENT (Olney): We don't know of any way that NMDA receptors would be involved in that kind of toxicity, but it is a fascinating possibility that you're putting your finger on a new type of excitotoxicity that hasn't been invented before.

COMMENT (Gibb): Right. We have found some very encouraging results, John; but as I said about the neuropeptide story, we can simulate or at least cause similar effects with NMDA as with methamphetamine on the increase in neuropeptides that Hanson has observed. We thought that with MK801 we can sort out those interrelationships better with the neuropeptides than we can neurotoxicity. It may be a little more physiologic than the neurotoxicity.

COMMENT (Ricaurte): John, just in relation to this whole question, we have envisioned that NMDA receptors are on DA terminals and hypothesized that if NMDA receptor activation is involved in the toxicity of amphetamines, we should be able to reproduce the toxicity by giving NMDA directly into the striatum. When you inject NMDA into the striatum, you see damage, but it appears to be nonspecific since DA levels per se are unaffected by high doses of NMDA. There is no question that the competitive and noncompetitive NMDA receptor blockers protect against methamphetamine toxicity. Just a few weeks ago at a New York Academy of Sciences meeting, John Marshall showed some very nice data obtained with in vivo microdialysis demonstrating that a compound like MK801 was very effective in interfering with methamphetamine-induced dopamine release; so indeed the basis of protection may involve NMDA receptors, perhaps by modulating DA release.

COMMENT (Gibb): Somehow I don't know which one comes first in the sequence, but obviously, it appears to be a component.

COMMENT (Seiden): There is no question that this result is reproducible.

Q (Landfield): What calcium channel blockers did you try?

A (Gibb): Nimodipine, which makes it worse. I have forgotten the others, but we tried quite a few.

COMMENT (Cho): I would just like to comment, because flunarizine is quite different chemically from some of the other calcium channel blockers. You are right; it is a dirty drug. But this goes back to what clearly represents my bias, but it seems that we ought to know the effects of these multiple drugs on the disposition of some of these compounds because part of the selectivity or part of the discrepancies that one might see could be due to the disposition or changes in the brain.

A (Gibb): We've certainly entertained that thought. It doesn't seem to fit with anything else. It could very well be that it is a disposition problem or it also has other effects on other systems. I think the thing that we have found fascinating about it is that it works selectively on one system and not the other. And that came as a bit of a surprise. It just so happens that it is the opposite of the MK801, and so it makes it kind of an interesting story.

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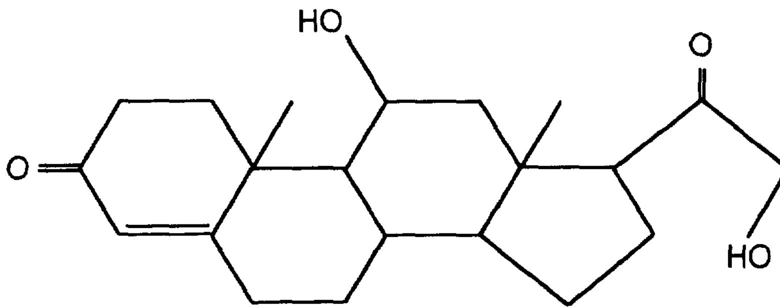
Neurotoxicity and Drugs of Abuse: Cannabinoid Interaction With Brain Glucocorticoid Receptors

Philip W. Landfield and J. Charles Eldridge

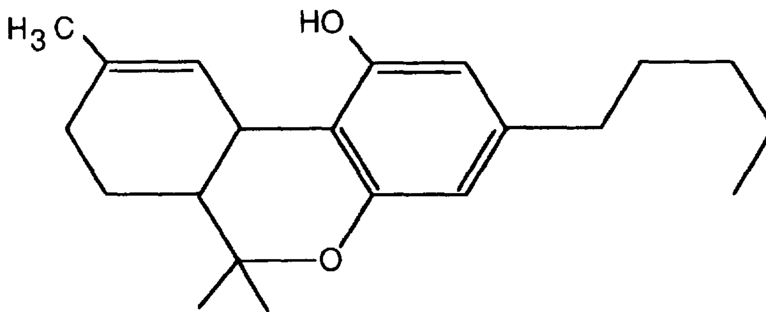
INTRODUCTION

Abuse of psychoactive pharmacologic substances continues to be one of the most difficult problems confronting the quality of human life in our society. Although there is wide recognition of the socioeconomic and personal problems created by alterations of behavior resulting from acute effects of drugs of abuse, evidence is presented here to indicate that further consideration should be given also to the deleterious physical effects of long-term exposure to these substances. It is important to recognize that many older users have been exposed chronically to drugs of abuse for many years and may believe that there is little risk in moderate but continued exposure. For example, results of a recent household survey conducted by the National Institute on Drug Abuse suggest that 4.8 percent of the entire US. population smoke marijuana at least once a month and that more than 3 million Americans use the drug "daily or almost daily" *(ADAMHA News* 1992). Because marijuana achieved a popular status during the 1960s it is likely that hundreds of thousands of middle-aged Americans have been exposing themselves continuously to this drug for more than two decades and perhaps their entire adult lives. Therefore, these users could be at significant health risk if drugs such as cannabinoids possess toxic effects with long-term exposure or if they are capable of interaction with the aging process.

The authors have recently described neurotoxic responses in the hippocampal regions of rats chronically administered Δ^9 tetrahydrocannabinol (THC), a major psychoactive cannabinoid, and have proposed that the mechanism for this toxicity may lie in similarity of cannabinoid structure and function to those of glucocorticoid (GC) steroids (Eldridge and Landfield 1990; Landfield et al. 1988; Eldridge et al. 1991) (figure 1). GC-mediated toxicity, particularly during brain aging, has been seen in a number of studies (Landfield and Eldridge 1991; Landfield et al. 1980) and evidence is reviewed in this chapter that brain-aging-like neurotoxicity can be exacerbated by continuous exposure to chemical substances that interact with GC-sensitive systems in hippocampus.



Corticosterone



THC

FIGURE 1. *Chemical structures of corticosterone and THC*

GC-MEDIATED NEUROTOXICITY IN STRESS AND AGING

It has been suggested that adrenal steroids could mediate aspects of peripheral aging. The classic studies of Selye and associates led them to propose that stress, by stimulating adrenal GC secretion, could augment the mammalian aging process (Selye and Tuchweber 1976). In addition, early studies of Robertson and Wexler demonstrated that Pacific salmon died normally from the catabolic effects of elevated GC secretion associated with spawning (Wexler 1976). Wexler (1976) further showed that elevated GC secretion enhanced cardiovascular and other disease conditions often associated with peripheral aging.

However, although genetic programing of cell death had been suggested as a possible underlying cause of neural aging (Finch 1976), there were few hypotheses in the mid-1970s that specifically addressed the mechanistic bases for irreversible degenerative change in aged neurons. However, it was recognized that the hippocampus and other brain regions are rich in GC receptors (McEwen et al. 1968; Stumpf and Sar 1975), and it was also known that the hippocampus is among the structures most vulnerable to normal brain-aging changes and to senile dementia of the Alzheimer's type (Tomlinson and Henderson 1976; Wisniewski and Terry 1973). Therefore, a series of studies was undertaken to test the hypothesis that GCs might modulate hippocampal aging. These studies found correlations between GC secretion and quantitative parameters of hippocampal aging (Landfield 1978; Landfield et al. 1978). It was further found that adrenalectomy could retard development of morphologic correlates of hippocampal aging (Landfield et al. 1981 a; Landfield 1987). Although one study has reported a loss of hippocampal neuron number in young animals administered high GC doses for a relatively short time (Sapolsky et al. 1985), it has remained difficult to perform long-term studies of elevated GC levels because of rapid decline of health of the animals under these conditions (Landfield 1987). Nevertheless, the findings have combined to suggest strongly that GCs can play a role in the vulnerability of the aging hippocampus (Landfield and Eldridge 1991; Landfield 1987; Sapolsky et al. 1985).

Additional studies of possible deleterious effects of endogenous GCs used indirect indicators of differential exposure to chronic stress (Meany et al. 1988; Uno et al. 1989). However, only recently were studies reported in which it was possible to maintain a mild, nondamaging stress for prolonged periods (Kerr et al. 1991). In these studies, it was found that several electrophysiologic and morphologic markers of brain aging were accelerated by chronic stress in rats of different ages. Furthermore, it appeared that the toxic impact of GCs on the hippocampus increased with aging, suggesting that an age-related "susceptibility factor" might increase the vulnerability of neurons to steroid-mediated toxicity.

Therefore, it is increasingly apparent that the development of pathology associated with brain aging, particularly as observed in the hippocampus, may be linked to the degree of exposure of the structures to GCs. The deleterious effects of this exposure arise apparently from persistent exposure to endogenous adrenal steroids and can be exacerbated by stressful environments, Conceivably, then, chronic exposure to xenobiotic substances such as drugs of abuse could also exert biologic or toxicologic effects on mammalian brain through the GC receptor system.

CHRONIC CANNABINOID EXPOSURE AND DEVELOPMENT OF NEUROPATHOLOGY

There are numerous reports in the literature that THC administration stimulates pituitary-adrenal secretion, including corticosterone (CORT) release, in animals (Dewey 1986; Martin 1986). Although accommodation of response has been

reported (Eldridge et al. 1991; Miczek and Dixit 1980), stimulation still can be elicited after extended chronic exposure (Landfield et al. 1988). Other studies have found that CORT stimulation following mild footshock was greater if animals were treated with THC (Jacobs et al. 1979) and that this enhanced secretion was likely caused by increased pituitary adrenocorticotropin (ACTH) release (Dewey et al. 1970). Further support for involvement of central nervous system mechanisms emerged from observations that barbiturate pretreatment reduced THC-stimulated CORT and ACTH release (Dewey et al. 1970; Barry et al. 1973).

Additional studies found that cannabinoid administration altered uptake of radiolabeled CORT in rat brain (Drew and Slagel 1973; Johnson et al. 1978), suggesting a mechanism involving GC receptors. It occurred to several researchers that similarities of cannabinoid chemical structure to steroids (figure 1) might cause the substances to substitute for GC binding in the brain (Martin 1986; Dewey 1986). This interaction could, for instance, inhibit GC-mediated negative feedback control of ACTH release, thereby activating pituitary-adrenal secretion. Moreover, because the hippocampus contains a dense population of GC receptors (McEwen et al. 1968; Stumpf and Sar 1975), and because this brain region is believed to participate in the homeostatic control of pituitary-adrenal secretion (De Kloet and Reul 1985; McEwen et al. 1986; Jacobson and Sapolsky 1991), it seemed conceivable that the hippocampus could be an important site of cannabinoid action. Therefore, it was hypothesized that chronic THC administration might induce hippocampal neuropathology, either by a direct agonist action through hippocampal GC receptors or by stimulating release of endogenous CORT to neurotoxic levels (Eldridge and Landfield 1990; Landfield et al. 1988; Eldridge et al. 1991).

Long-term administration studies have yielded data highly consistent with this hypothesis (Landfield et al. 1988). In these studies, it was observed that THC-treated rats (8 to 10 mg/kg x 5 days/week x 8 months) exhibited decreased neuronal density and increased glial cell reactivity in the hippocampus. The changes appeared similar in degree to those observed in normally aged rats of 24 months or older (Landfield et al. 1981*b*). However, the THC-treated animals were only 11 to 12 months old at sacrifice, suggesting that THC might have accelerated brain-aging-like activities. These changes are described further below.

Assessment of Neurotoxicity

The paradigm of coupling long-term drug or hormone treatment with quantitative neuroanatomy was developed initially to test hypotheses on physiologic factors that may modify the development of morphological correlates of brain aging as well as to study mechanisms that govern gradual structural remodeling in the adult mammalian brain. It was clear that experimental treatments such as hormonal manipulations or pharmacological agents would need to be maintained for extended periods (e.g., 20 to 30

percent of the lifespan or 5 to 9 months in rats) and that extremely sensitive techniques for measuring morphological and behavioral correlates of brain aging would be required (see Landfield 1982 for methodological issues). As a result, sensitive and reliable indices for quantifying subtle differences in brain organization have been developed. The accuracy and reliability of the methods are greatly enhanced by measurement of multiple morphometric variables because these variables cross-validate one another and tend to cancel out random variances (Landfield 1962).

Long-Term Exposure to THC

Using similar paradigms and procedures, long-term studies on the effects of chronic treatment with THC were undertaken, based on extensive quantitative light and electron microscopic (EM) studies. As mentioned, in the first study (Landfield et al. 1988), chronic THC decreased hippocampal neuronal density but not synaptic density (although another group of investigators has found altered synaptic density in THC-treated rats [Scallet et al. 19871). THC also increased the percentage of astrocyte cytoplasm occupied by inclusions at the EM level (Landfield et al. 1988). In a second long-term (8 months) study, effects were replicated, and it also was found, at the EM level, that THC administration increased the cytoplasmic volume occupied by Golgi apparatus (Cadwallader et al. 1989). These results were consistent with the possible stimulation of genomic activity by GC agonist-like activation in these neurons.

The similarity of the THC-dependent pattern of neurotoxicity to the aging/ CORT-associated neuropathology pattern suggested an interaction of THC with steroid systems in the induction of the pathological effects. In addition, during acute restraint stress, plasma levels of both ACTH and CORT were significantly elevated in rats treated chronically (4 months) with THC, compared with those given pluronic vehicle alone or saline (Landfield et al. 1988) which confirmed numerous reports, discussed above (Dewey 1986; Martin 1986), that THC stimulates CORT release. The results also demonstrated that the endocrine alterations did not completely adapt with prolonged exposure and therefore could be sufficient to advance aging-like effects.

CANNABINOID INTERACTION WITH BRAIN GC RECEPTORS

As a result of suggestions that cannabinoids might be capable of direct interaction with GC receptors in the brain, two possible mechanisms were hypothesized: On the one hand, pituitary-adrenal activation could result from cannabinoid *antagonism* of normal GC negative feedback suppression of ACTH release, leading to increased CORT secretion; on the other hand, cannabinoids could express direct *agonist* activity through GC receptors in the hippocampus, to imitate or promote the same neuropathologic changes that appear to be induced by CORT (Landfield 1987; Sapolsky et al. 1985; Kerr et al. 1991). Either of these hypothesized mechanisms likely would require direct interaction of cannabinoids with GC-receptor-binding events in brain.

These hypotheses were tested by preparing cytosol from hippocampal tissue of adrenalectomized (ADX) rats (to remove all endogenous corticosteroids) and incubating the cytosols with radiolabeled dexamethasone (DEX) plus or minus various potential competing agents (for additional methodologic details, see Eldridge et al. 1991; Eldridge and Landfield 1990). With inhibition of tracer binding to receptors set at 100 percent by determination of displacement with 100-fold molar excess unlabeled DEX, it was observed that 100-fold molar excess THC displaced 32 percent of tracer binding, whereas cannabidiol (CBD), a nonpsychoactive constituent of marijuana, displaced only 16 percent of specific radiolabeled DEX binding sites.

The rat hippocampus contains two specific types of GC receptor, and DEX binds to both types in vitro (Reul and De Kloet 1985; Landfield and Eldridge 1989). In our studies, incubation of hippocampal cytosol with [^{3}H]-DEX plus 100-fold excess of RU-28362 (RU), a specific type II or GC receptor ligand (Hermann et al. 1987; Raynaud et al. 1984), caused displacement of 81 percent of total binding capacity. Additional incubations of RU plus either THC or CBD failed to displace more receptor binding than did RU alone, indicating that cannabinoid displacement more likely affected the type II population. Interaction with type I (mineralocorticoid receptor) binding would have appeared as a further displacement when THC or CBD was added to RU. Similar results to these were obtained when cytosols incubated with [^{3}H]-DEX plus or minus RU, THC, or CBD were layered onto 5 to 20 percent sucrose gradients and centrifuged to separate various binding components by molecular weight. Cannabinoid displacement of GC binding occurred in the 8S region, where the cytoplasmic GC receptor migrates.

Studies of the kinetics of cannabinoid competition also were performed by incubation of cytosol with a range of doses of radiolabeled DEX in a Scatchard-type assay (Eldridge and Landfield 1990; Eldridge et al. 1989*a*). Plots of the data of high-affinity receptors should be linear, with the x-intercept indicating binding capacity, or B_{max}. Inhibitor agents, whether competitive or noncompetitive inhibitors, shift the plot in relation to their ability to compete against tracer binding. As in the previously described studies, RU-28362 competed against binding to type II sites (184 fmol/mg cytosol protein vs. a total capacity of 210 fmol/mg; type I=the difference of 26 fmol/mg). Again, THC at 100-fold molar excess was able to displace a significant, but incomplete, portion of tracer binding (lower B_{max}), and the Scatchard plot with THC added was parallel to that of either total receptors or the type II fraction (figure 2).

If THC binds to the full population of type II GC receptors, a parallel slope and lower B_{max}, would be viewed as evidence that the THC interaction was noncompetitive in nature. If the competition between competitor (THC) and tracer (DEX) had occurred at the same binding site, the Scatchard plot would have demonstrated the same B_{max} as seen with incubation with unlabeled DEX, but the slope (affinity) would have been different. However, no change in affinity was seen in the THC plot; the x-intercept was lower, indicating that

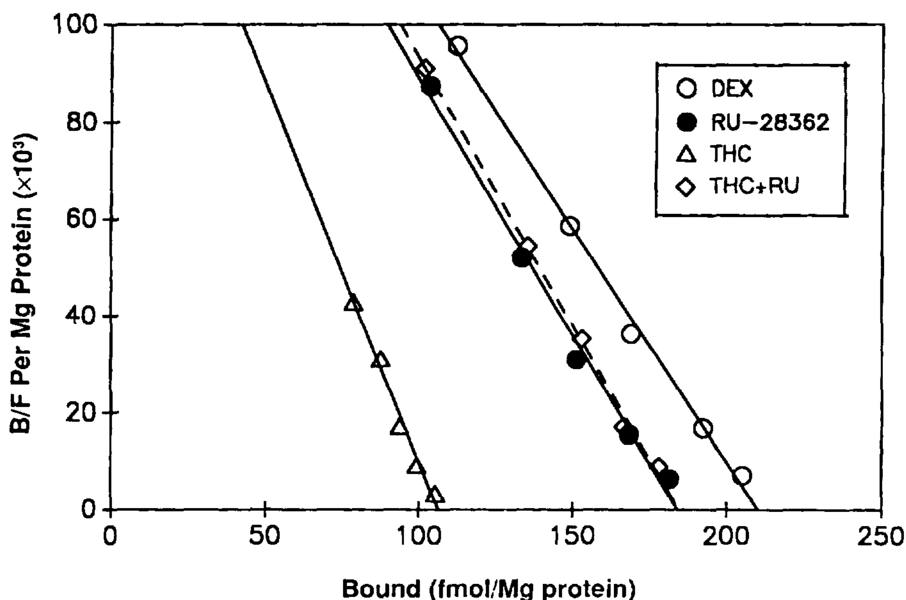


FIGURE 2. *Scatchard plot of cannabinoid competition with [^{3}H]-DEX binding to hippocampal GC receptors. Aliquots of pooled cytosol prepared from ADX rats (methodologies as described in Eldridge and Landfield 1990 and Eldridge et al. 1989*a*) were incubated in vitro with 1.25 to 20 nM radiolabeled DEX alone or plus 100-fold molar excess of unlabeled DEX, RU-28362, THC, or CBD. DEX plot indicates total specific displacement (210 fmol/mg protein), and RU plot indicates type II receptor displacement (184 fmol/ mg). The slope of the THC plot (K_d=2. 18 nM) was approximately parallel to those for DEX (3.11 nM) and RU (2.85 nM), but THC displaced fewer sites (106 fmol/mg). A combination of RU and THC, each at 100-fold molar excess over tracer concentration, produced a plot not different from that produced by RU alone, Cannabidiol displacement of [^{3}H]-DEX was very slight, not linear, and could not be plotted.*

SOURCE: Eldridge, J.C.; Murphy, L.L.; and Landfield, P.W. Cannabinoids and the hippocampal glucocorticoid receptor: Recent findings and possible significance. *Steroids* 56:226-231, 1991. Reprinted with permission of Butterworth-Heinemann, Inc.

the capacity for radiolabeled DEX binding was reduced in the presence of THC. Such a plot suggests a “noncompetitive” type of THC inhibition, wherein the competitor associates with an allosteric site, distinct from the hormone binding domain, that nevertheless alters the ability of the steroid to bind at its domain.

There is increasing evidence of allosteric binding sites on steroid receptors. For example, Svec (1988) has described noncompetitive effects on corticosteroid receptor binding by progesterone as well as by nonsteroidal agents. However, substantially more work is necessary before the nature of THC interaction with GC receptors is fully clarified.

THC-Mediated Down-Regulation of GC Receptors

One additional and critical test of the ability of THC to alter GC receptors was conducted, which, in several ways, provided some of the clearer evidence of a functionally significant interaction of THC and brain GC receptors. It is well established that elevated secretion or administration of large doses of CORT down-regulates GC receptor levels in the hippocampus (De Kloet and Reul 1985; McEwen et al. 1986; Tornello et al. 1982; Eldridge et al. 1989*b*). When GC receptor content was analyzed in ADX rats maintained on a low level of replacement steroid and given 10 mg/kg THC for 14 days (a dose that rats tolerated in our long-term studies), down-regulation of GC receptor binding was likewise observed in the THC-treated animals. Type I receptor content was not affected. Previous studies have indicated that CORT administration can down-regulate both receptor types (De Kloet and Reul 1985), so these results appear to provide strong support for a functionally relevant and specific in vivo effect of THC on the type II GC receptor. The occurrence of the response in ADX animals suggests that the THC effect was mediated by direct interaction with GC receptors rather than through secondary release of adrenal steroids.

The authors and colleagues have reported that down-regulation of brain GC receptors was significantly impaired in aged animals exposed to daily stress for 6 months (Eldridge et al. 19896) and that up-regulation of GC receptors that normally occurs following adrenalectomy also was impaired in aged rats (Eldridge et al. 1989*a*). The fact that hippocampal GC receptors down-regulate less in intact aged rats has raised the possibility that this could increase the vulnerability of hippocampal neurons to CORT-related degeneration during aging (Landfield and Eldridge 1991). It is also conceivable that repeated lifelong activation of brain GC receptors leads over time to reduced plasticity and, thus, to increased vulnerability and neuronal loss. It therefore seems possible that chronic exposure to THC could accelerate this process, perhaps by increasing the vulnerability of hippocampal neurons.

Agonist or Antagonist Effects

Further evidence demonstrating direct agonist-like effects of cannabinoids on GC-mediated response systems has recently been observed in the authors' laboratories. Levels of mRNA for glial fibrillary acidic protein, which has been shown to be specifically inhibited by GCs (Nichols et al. 1990), were also found to be reduced in the hippocampus of ADX rats treated with THC (Eldridge et al. 1992). In addition, levels of calbindin-D_{28K}, which have been shown to increase specifically in hippocampus following GC exposure (lacopino and Christakos

1990), have been found in our laboratories to be increased in the hippocampus of ADX rats treated with THC (Geddes et al. 1992).

Based on these findings, there appears to be considerable support for the possibility that THC, which affects the neuroendocrine stress response, also interacts directly with brain GC receptors and modulates the impact of corticosteroids on the brain. It is clear that a substance need not be a steroid in molecular structure to regulate GC receptor binding. Therefore, one result of chronic exposure to cannabinoids could be allosteric modulation of brain GC receptors such that one or more of the following occurs: (1) the effects of CORT activation are mimicked; (2) endogenous CORT binding to GC receptors is enhanced; (3) regulatory plasticity of GC receptors is impaired, making neurons more susceptible to the toxic effects of CORT; (4) negative feedback control of ACTH is partially blocked, resulting in higher CORT levels and leading to greater neuronal degeneration; or (5) neuroendocrine control mechanisms unrelated to GC receptors are altered, resulting in a persistent hyperactivity of stress responses and higher CORT secretion. These possibilities are, of course, not mutually exclusive, nor would they represent all possible means through which drugs of abuse may alter or mimic CORT impact on the brain.

INTERACTIONS OF OTHER DRUGS OF ABUSE WITH STRESS SYSTEMS AND INDUCTION OF NEUROPATHOLOGY

To date, clear-cut quantitative neuroanatomical evidence of brain-aging-like neuron loss in the hippocampus, or of brain GC receptor modulation, has been found for chronic exposure to only one drug of abuse, that is, THC. However, other types of neuropathology have been seen with several drugs of abuse, particularly stimulants such as methamphetamine.

With only a few exceptions (e.g., in Seiden et al. 1976), neuropathological or synaptic remodeling effects of major addicting drugs have not been examined in long-term paradigms similar to those in which THC effects have been found (e.g., 7- to 8-month exposure [Landfield et al. 1988]); the longest treatment period in a neuropathology-cocaine study appears to have been 21 days (Seiden and Kleven 1989). Thus, if other drugs of abuse act in part analogously to THC and/or interact with gradual brain-aging-like neurotoxic mechanisms or if they induce wholly different, but nevertheless subtle, synaptic remodeling changes, their brain-structural effects might not be detectable in animals until after several months of exposure.

There also is growing evidence that drugs of abuse other than THC are capable of interaction with brain neuroendocrine stress systems. For example, several studies have shown that cocaine, amphetamines, and methylenedioxymethamphetamine (MDMA) are strong stimulants of ACTH and CORT release, under a variety of conditions; moreover, the site of action appears to lie in the brain (Moldow and Fischman 1987; Rivier and Vale 1987).

There are also strong effects of morphine and other opioids on the adrenal-pituitary axis, but the pattern of these effects is controversial; some studies have found a decrease, whereas others find an increase, depending on experimental conditions. This discrepancy may arise possibly because there are multiple mechanisms and sites of opioid modulation of CORT release, one inhibitory and one stimulatory (Bird et al. 1987; Cetera et al. 1988; Nikolarakis et al. 1987). It is also clear that brain serotonin systems are intimately involved in regulation of the pituitary-adrenal stress response (Azmetia and McEwen 1974; Jhanwar-Uniyal et al. 1987; Feldman et al. 1984; Lorens and Van de Kar 1987) and that amphetamines, cocaine, MDMA, and morphine strongly modify serotonin systems (Seiden and Kleven 1989; Cunningham and Lakosky 1988; Dworkin and Smith 1987). Thus, a wide range of drugs of abuse interacts with the brain control mechanisms of the stress response.

In addition, there is recent intriguing evidence that drugs with some abuse potential can directly modulate brain corticosteroid receptors. For example, the tricyclic antidepressant imipramine, which, like cocaine, is a reuptake blocker at catecholaminergic terminals, was found to alter GC binding in cells of locus coeruleus and the raphe nuclei (Kitiyama et al. 1988). In addition, MDMA reduced both serotonin and type II GC receptors in the striatum (Lowy et al. 1989). These effects of both imipramine and MDMA were independent of plasma CORT release and may reflect secondary modulation of GC receptors by activation of catecholamine or serotonin receptors. These results are consistent with the possibility that monoamine receptor activation can directly regulate corticosteroid receptor concentration in the same neurons (Kitiyama et al. 1988; Lowy et al. 1989). Although we have suggested that THC interacts with GC receptors directly (Eldridge and Landfield 1990; Landfield et al. 1988; Eldridge et al. 1991) and its molecular structure bears some similarity to that of steroids (Martin 1986), it also should be noted that similarity of molecular structure is clearly not a requisite for the potential modulation of brain GC receptors by drugs of abuse that activate the stress response. That is, corticosteroid receptors apparently contain at least one allosteric site at which binding of ligands can be modified, both by other steroids (e.g., progesterone and corticosterone) and by other agents (e.g., ketoconazole) that bear no apparent similarity to steroids (Svec 1988).

Thus, it seems clear that a number of drugs of abuse can modulate GC receptors, either by direct interaction or by secondary effects following monoamine receptor activation. Furthermore, a range of drugs of abuse modulate the adrenal-pituitary system that can, in turn, feed back onto brain GC receptors. For these reasons, there appear to be multiple pathways through which drugs of abuse can modulate the brain's response to adrenal stress hormones. In addition, there are reports that some drugs of abuse in addition to THC can induce neurotoxicity in selected systems (Seiden et al. 1976; Nwanzee and Johnson 1981; Ricuarte et al. 1983, 1984; Lorenz 1981). Therefore, it is conceivable that some aspects of this neurotoxicity may also be mediated by effects on GC receptors.

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DISCUSSION

Q: Did you see anything in dentate granule cells with THC?

A (Landfield): Well, we didn't measure them because they are generally very resistant to the loss of cells during brain aging.

Q: What about extrapolating your data to the humans?

A: Chronic stress is bad for you; chronic THC is bad for you; aging is bad for you.

Q: So, should we retire early?

A: That might be more stressful for some.

Q: Does your THC regimen remove the thymus?

A: I don't know. I think it does. But, no, I will take it back. We considered measuring it, and I think we looked at a few animals, but we don't have systematic data on thymus involution.

Q: Have you had a chance to look at other receptors? I guess my question is that THC is a rather dirty compound. Does it interact with a number of receptors, not just glucocorticoid receptors?

A: There is a receptor termed the “cannabinoid receptor” that is also bound by THC. My colleague, Chuck Eldridge, is now looking at the interaction of glucocorticoid receptors with cannabinoid-like, or CP compounds. There is a little bit of interaction, but it is very dependent upon whether it is studied in membrane or cytosol. The cannabinoid receptor is membrane bound, and the glucocorticoid receptor is cytosolic. However, it is certainly conceivable that there is more than one THC site In the brain, given the multiplicity of effects that THC has. I think it still needs to be determined more clearly whether the CP site or the GC site is the site of greater psychoactive relevance, and in which proportion. I suspect that the GC site is important for stress reduction effects of THC, and certainly it may have neurotoxic relevance; but whether it has other psychoactive relevance is something that we will have to study in the future.

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Nitric Oxide as a Mediator of Neurotoxicity

Ted M. Dawson, Valina L. Dawson, and Solomon H. Snyder

INTRODUCTION

Nitric oxide (NO) is a newly identified neuronal-messenger molecule, first recognized as endothelium-derived relaxing factor (EDRF) and as a modulator of the tumoricidal and bactericidal actions of macrophages (Marletta 1989). The brain NO synthase (NOS) has been purified to homogeneity as a 150-kilodalton protein with an absolute requirement for calcium, calmodulin, and nicotinamide-adenine dinucleotide phosphate (NADPH) (Bredt and Snyder 1990). It has been cloned and has sequence homology to cytochrome P-450 reductase (Bredt et al. 1991*a*). The macrophage NOS also has been purified to homogeneity and is a dimer of molecular weight 250 kilodalton (Stuehr et al. 1991). The macrophage enzyme differs from the brain/endothelial enzyme in that it does not require calcium and calmodulin but has a requirement for tetrahydrobiopterin (Tayeh and Marletta 1989; Kwon et al. 1990; Yui et al. 1991; Stuehr et al. 1991). A physiologic role for NO in the brain and nervous system has been established by demonstrations that arginine derivatives, which are potent and selective inhibitors of NOS, block neuronally mediated relaxation of the intestine (Bult et al. 1990; Boeckxstaens et al. 1991) and stimulation of cyclic guanosine monophosphate (cGMP) formation by glutamate in the cerebellum (Garthwaite et al. 1988, 1989; Bredt and Snyder 1989). NOS has been immunohistochemically localized to discrete regions of the brain and neurons in the periphery. It is localized to populations of medium-to-large aspiny neurons in the cerebral cortex and corpus striatum, basket and granule cells of the cerebellum, pedunculopontine nucleus, and other areas (Bredt et al. 1990; Dawson et al. 1991*a*), In the periphery, it is localized to ganglion cells and their fibers in the adrenal medulla, myenteric plexus neurons of the intestine, and nerve fibers in the posterior pituitary that receive projections from NOS-containing neurons in the supraoptic and paraventricular hypothalamic nuclei (Bredt et al. 1990; Dawson et al. 1991*a*).

NOS AND NEURONAL NADPH-DIAPHORASE

Dawson and colleagues (1991*a*) have shown that the distribution of NOS neurons matches precisely with the distribution of NADPH-diaphorase-containing neurons,

NADPH-diaphorase-containing neurons were first identified histochemically by Thomas and Pearse (1984, 1961) by the reduction of nitroblue tetrazolium by NADPH to a formazan precipitate. Much interest in the function of NADPH-diaphorase stems from observations that these neurons are selectively resistant to the degeneration in Huntington's disease and survive ischemia as well as destruction of neuronal tissue by excitatory amino acids (Ferrante et al. 1985; Uemura et al. 1990; Beal et al. 1986; Koh et al. 1986; Koh and Choi 1988).

During our initial studies of the distribution of NOS, the authors and coworkers did not observe close colocalization with any known neurotransmitters or neuronal markers. However, it became clear that the distribution of NOS seemed to parallel the distribution of NADPH-diaphorase-containing neurons. In the brain and periphery, NOS-containing neurons matched precisely with the distribution of NADPH-diaphorase-containing neurons, In the brain, NOS is colocalized with neuropeptide Y (NPY) and somatostatin-containing neurons in the striatum and cortex (Dawson et al. 1991*a*), In the striatum, these are the neurons that survive in Huntington's disease (Ferrante et al. 1985). In contrast, in the pedunculopontine nucleus, NOS-containing neurons colocalize with choline acetyltransferase (ChAT)-containing neurons. In the pedunculopontine nucleus, NOS-containing neurons do not colocalize with the somatostatin- and NPY-containing neurons, and in the striatum, NOS does not colocalize with ChAT-containing neurons (Dawson et al. 1991*a*).

In the periphery, NOS colocalizes with NADPH-diaphorase-containing nerve fibers in the posterior pituitary as well as with the NOS-containing neurons of the supraoptic and paraventricular hypothalamic nuclei. In the adrenal medulla, NOS colocalizes with NADPH-diaphorase-containing ganglion cells. The adrenal cortex displays NADPH-diaphorase staining but no NOS immunoreactivity. Several NADPH-requiring enzymes of the steroid biosynthesis pathway may account for NADPH-diaphorase staining observed here. In the intestine, NOS-containing neurons colocalize with NADPH-diaphorase-containing myenteric plexus neurons. These neurons probably account for the relaxation of the intestine and stomach by NO (Bult et al. 1990; Boeckxstaens et al. 1991).

By utilizing oligonucleotide probes to the cDNA for NOS, Bredt and colleagues (1991*b*) have also colocalized the message for NOS to NADPH-diaphorase neurons in the brain. The striking colocalization of NOS with NADPH-diaphorase suggests that NOS catalytic activity accounts for NADPH-diaphorase staining. By transfecting the whole open-reading frame of the cDNA for NOS into human kidney 293 cells, encoded protein and catalytic activity were demonstrated in these cells (Dawson et al. 1991*a*). These transfected cells exhibit NADPH-diaphorase staining that is proportional to the amount of expressed NOS immunoreactivity. In addition, the relative amount of NOS immunoreactivity and NADPH-diaphorase staining is identical in individual neurons of several regions of the brain (table 1).

TABLE 1. *Comparison of NOS immunoreactivity and NADPH-diaphorase staining*

Tissue	NOS	NADPH-Diaphorase
Recombinant NOS protein		
Mock transfected cells	0	0
NOS transfected cells (1 μg cDNA)	4+	4+
NOS transfected cells (10 μg cDNA)	8+	8+
Cerebellum		
Basket ceils	2+	2+
Granule cells	2+	2+
Purkinje cells	0	0
Cortex		
Medium-large aspiny neurons	4+	4+
Hypothalamus		
Paraventricular nucleus	3+	3+
Supraoptic nucleus	3+	3+
Pedunculopontine tegmental nucleus	5+	5+
Retina		
Amacrine cells	2+	2+
Ganglion cells	1+	1+
Striatum		
Medium-large aspiny neurons	4+	4+

NOTE: In 4 to 6 separate experiments in which 20 to 40 neurons were examined per region, the density of NOS immunoreactivity is identical to NADPH-diaphorase staining (1+=low; 8+=high). In kidney cells transfected with NOS cDNA, the ratio of immunoreactive NOS and NADPH-diaphorase staining is identical to that seen in positive neurons.

SOURCE: Dawson et al. 1991*a*

These findings establish that NOS is responsible for NADPH-diaphorase staining of neurons.

Another approach to establish that NOS accounts for NADPH-diaphorase staining would be to purify the NADPH-diaphorase protein and determine whether it is

identical to the NOS protein. However, NADPH-diaphorase activity measured in homogenates may not reflect the enzyme monitored by histochemical staining. In efforts to purify NADPH-diaphorase by monitoring nitroblue tetrazolium reduction, we have observed multiple protein bands that are distinct from NOS. Hope and colleagues (1991) also have observed substantial NADPH-diaphorase catalytic activity that could be separated from NOS activity. However, Hope and colleagues (1991) did show that purified NOS does possess NADPH-diaphorase activity that is similar to our own observations. As such, histochemistry reveals enzyme activity that is highly concentrated in discrete neurons, whereas the catalytic activity of homogenates may reflect the contribution of multiple enzymes with “diaphorase” activity.

NO AND NEUROTOXICITY

The identity of NADPH-diaphorase-containing neurons with NOS suggested that NO may be involved in modulating neurotoxicity, because these neurons survive a variety of toxic insults. Dawson and colleagues (1991*b*) used rat primary cortical cultures as described by Koh and Choi (1988) to investigate the effects of inhibitors of NOS on glutamate neurotoxicity. Neurotoxicity was assessed by the exclusion of trypan blue or by the measurement of lactate dehydrogenase. In the primary culture system, N-methyl-D-aspartate (NMDA) displays a toxic LD_{50} of 280 µM. A 300 µM concentration of NMDA consistently destroys approximately 60 percent of the neurons (figure 1). Simultaneous application of 300 µM NMDA with 100 µM nitroarginine (N-Arg), a NOS inhibitor, attenuates NMDA-induced cell death by approximately 70 percent (figure 1). The addition of 1 mM L-arginine (L-Arg), the endogenous substrate for NOS, reverses the protective effects of N-Arg (figure 1). To further ascertain whether NO is involved in NMDA neurotoxicity, 500 µM of reduced hemoglobin, which binds NO, was added simultaneously with 300 µM NMDA (figure 1). Hemoglobin completely prevents NMDA toxicity. The 500 µM concentration of reduced hemoglobin employed is similar to that required for reduced myoglobin to prevent macrophage-mediated cell death (Stuehr and Nathan 1989).

Cortical cultures possess NOS catalytic activity and protein that are approximately 10 percent that of cerebellar levels (figure 2). In addition, NMDA can stimulate the formation of NO in cortical cultures. The formation of cGMP can be used as an indirect measure of NO formation, because NO binds to the heme moiety of guanylate cyclase and subsequently activates the enzyme (Bredt and Snyder 1989). NMDA stimulation of cGMP levels in cultures is antagonized by N-Arg, and this antagonism is competitively reversed by L-Arg. Superoxide dismutase, which stabilizes NO, enhances NMDA stimulation of cGMP. Hemoglobin, which complexes NO, prevents NMDA-induced cGMP formation, Thus, as in cerebellar slices, NO stimulates cGMP formation in cortical cell cultures (Dawson et al. 1991*b*).

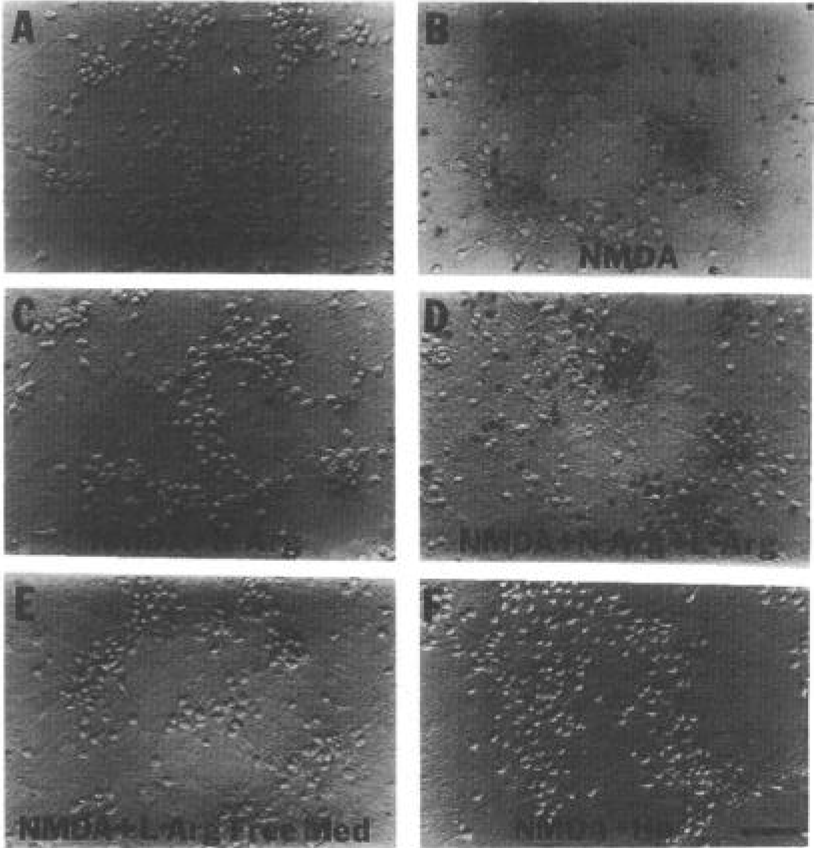


FIGURE 1. *Hoffman modulation photomicrographs of primary cortical cultures illustrating the effect of manipulation of NOS on NMDA neurotoxicity. Panel A: Control well of primary cortical neurons. Panel B: 300 μM NMDA+ 10 μM glycine consistently yields 60 to 70 percent neuronal death as determined by trypan blue exclusion. Dead neurons appear as black dots, and viable neurons remain unstained. Panel C: 100 μM N-Arg protects* against NMDA toxicity. Panel *D:* 1 mM L-Arg reverses *N-Arg neuroprotection, thus demonstrating that NMDA toxicity is mediated via NO. Panel E: Depletion of the culture media of L-Arg* with arginase or *growing* the *cells in an L-Arg-free* media *completely attenuates neurotoxicity, indicating an L-Arg dependence for NMDA-mediated cell death. Panel F: 500 μM reduced hemoglobin (Hb) that inactivates NO attenuates* NMDA *neurotoxicity; thus, NO is released to mediate* its *toxicity. Bar=100 μM.*

SOURCE: Dawson et al. 1991*b*

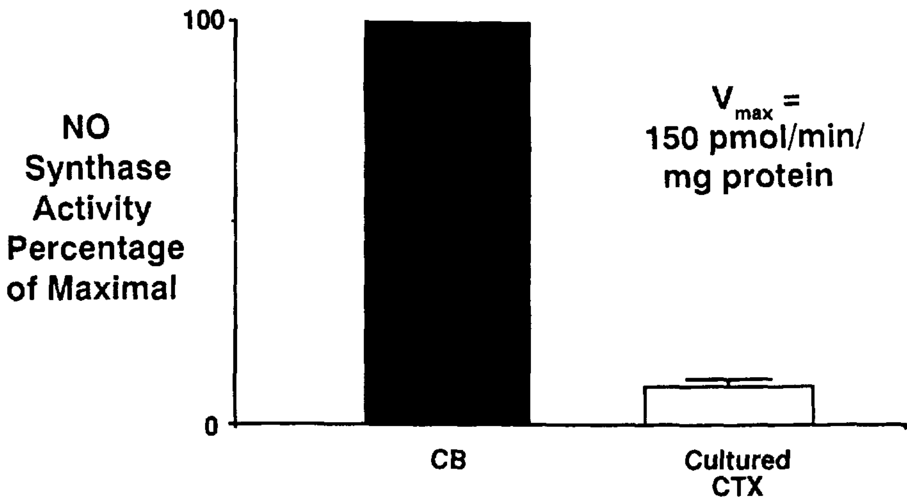


FIGURE 2. *NOS catalytic activity was assessed by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline in homogenized primary cortical (CTX) neuron* cultures and compared *with cerebellar (CB) homogenates. NOS catalytic* activity *in CTX was approximately 1/10 that of CB. Similar results were obtained by Western blot analysis.*

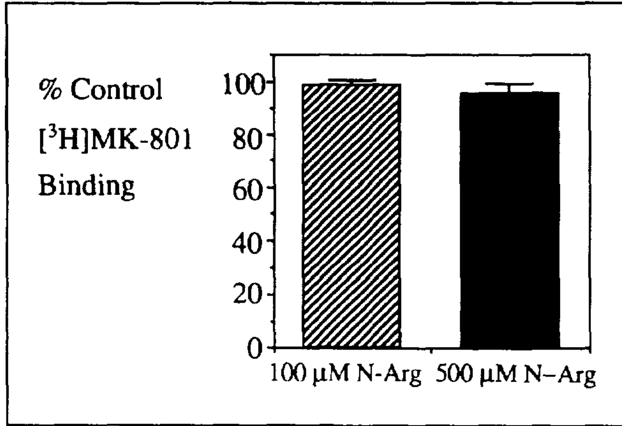
SOURCE: Dawson et al. 1991*b*

N-Arg inhibits NMDA-induced cell death with an EC_{50} of 20 μM. Methylarginine (M-Arg), another NOS inhibitor that is less potent than N-Arg, inhibits NMDA-induced cell death with an EC_{50} of 170 μM (Dawson et al. 1991*b),* N-Arg does not act directly on NMDA receptors because N-Arg has no effect on [^{3}H]MK-801 binding or on NMDA-induced currents measured in cortical cultures by whole-cell patch-clamp analysis (figure 3).

Because L-Arg is able to reverse the neuroprotective effects of N-Arg against NMDA neurotoxicity, we wondered whether L-Arg plays a role in NMDA neurotoxicity. To determine that fact, arginase was added in amounts that completely degraded L-Arg or treated sister cultures for 24 hours in a medium without L-Arg, a procedure that fully depletes cells of L-Arg (Sessa et al. 1990). In both preparations, 300 μM NMDA no longer causes cell death, and the LD_{50} of NMDA is increased more than twentyfold to 7.5 mM. Furthermore, there is an absolute requirement of 100 mM L-Arg to obtain maximal NMDA neurotoxicity.

Although NMDA-receptor activation presumably accounts for the majority of neurotoxicity associated with synaptically released glutamate, activation of

N-Arg Does Not Affect [^{3}H]MK-801 Binding to the NMDA Receptor Channel in Cortex



N-Arg Does Not Affect NMDA-Induced Currents in Cultured Cortical Neurons

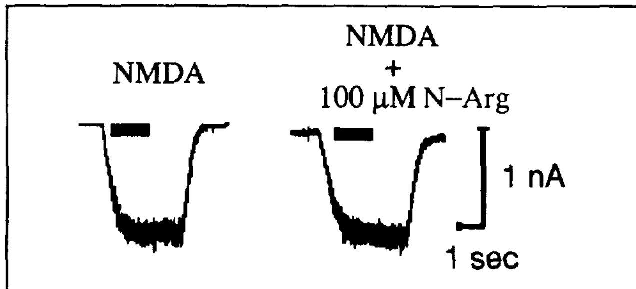


FIGURE 3. *N-Arg has no effect on [^{3}H]MK-801 binding or NMDA currents as assessed by whole-cell patch-clamping.*

SOURCE: Dawson et al. 1991*b*

quisqualate and kainate receptors may also play a role (Koh et al. 1990). The effects of N-Arg on cytotoxicity induced by quisqualate, kainate, and NMDA were compared (table 2). Quisqualate neurotoxicity is diminished somewhat by treatment with high concentrations of N-Arg, perhaps because at these concentrations quisqualate influences NMDA receptors (Koh et al. 1990). N-Arg provides no protection against kainate-induced cell death. Effects of glutamate and its analogs are similar in cultures from the caudate, hippocampus, and cerebral cortex.

The authors evaluated the specificity of L-Arg in reversing N-Arg-neuroprotective effects against NMDA toxicity by examining various analogs of L-Arg. Homoarginine, which can serve as a precursor of NO but has less affinity for NOS than L-Arg, partially reverses the effects of N-Arg. The stereoisomer D-arginine is much weaker than L-Arg. These results establish the specificity for L-Arg reversal of N-Arg neuroprotection against NMDA neurotoxicity.

TABLE 2. *Inhibition of glutamate neurotoxicity by N-Arg and reversal by L-Arg in primary neuronal cultures*

Compound	% Cell Death Cortex
300 µM NMDA	62.0±2.5
+100 µM N-Arg	20.3±3.0*
+N-Arg+1 mM L-Arg	64.6±6.5
500 µM quisqualate	60.7±4.1
+500 µM N-Arg	41.5±3.6*
+N-Arg+5 mM L-Arg	63.0±4.5
100 µM kainate	83.9±4.3
+500 µM N-Arg	83.0±4.1

NOTE: Values represent the means±SEM for n>6, n<30. Cell death was determined by 0.4 percent trypan blue exclusion by viable cells. Significant overall F values were obtained by using a one-way, between-groups analysis of variance. Specific comparisons on all possible pairwise combinations were made with the Spjotvoll-Stoline post hoc test.

*p<0.05

SOURCE: Dawson et al. 1991*b*

Sodium nitroprusside, which spontaneously releases NO, was used to determine the direct effect of NO on cortical cells. A 5-minute application of sodium nitroprusside elicits cell death in a concentration-dependent fashion with a 50-percent maximal response at 50 μM and enhancement of cGMP levels in cortical cells at a similar potency. The influence of sodium nitroprusside on cGMP levels as well as cytotoxicity appears to involve NO, because these effects are blocked by hemoglobin. In addition, the dose-response relationship and time-course of NO-mediated neuronal death parallels NMDA-mediated neurotoxicity (Dawson et al., in press).

The experiments described above indicate that NO mediates glutamate neurotoxicity in neuronal cultures. Glutamate-receptor-mediated cell death is prevented by inhibitors of NOS whose protective effects are proportional to their potencies as inhibitors of NOS. The inhibition of cell death is competitively reversible by L-Arg and is exerted also by various arginine derivatives in proportion to their activities as substrates for NOS. Removal of arginine from the culture media by arginine-free media or by arginase also prevents NMDA-induced cell death, demonstrating an absolute requirement of L-Arg for NMDA-induced neurotoxicity. Furthermore, sodium nitroprusside, which generates NO, elicits cell death in a concentration-dependent fashion that parallels the formation of cGMP. Reduced hemoglobin, which complexes NO, prevents both NMDA- and sodium nitroprusside-induced neurotoxicity. N-Arg also diminishes infarct volume following focal ischemia in mice, thus establishing the physiologic relevance of NO in neurotoxicity (Nowicki et al. 1991).

NO previously has been shown to mediate cytotoxicity resulting from activated macrophages that kill tumor cells and bacteria via NO. The exact source of neurotoxic NO is not yet definitively established. Neurons that contain NOS are a likely source, whereas microglia, which may contain a macrophage-like NOS, are alternative candidates. If neuronal NOS mediates glutamate neurotoxicity, then destruction of NOS-containing neurons should attenuate glutamate neurotoxicity. Choi and coworkers (Koh and Choi 1988; Koh et al. 1986) have shown that NADPH-diaphorase neurons, which are now known to be NOS neurons, in primary cortical cultures resist NMDA neurotoxicity but are extremely susceptible to kainate or quisqualate toxicity. Low-dose quisqualate treatment of the cultures, which selectively kills NOS/NADPH-diaphorase neurons, subsequently reduces the toxicity of NMDA, implying that these neurons mediate NMDA killing of other neurons (Dawson et al., in press). Furthermore, neuronal cell death tends to occur around the processes of NOS neurons that ramify extensively, contacting the majority of neurons in the culture even though NOS neurons comprise only about 2 percent of neurons in the culture. Ontogeny studies show that the appearance of NOS immunoreactivity coincides with the development of NMDA toxicity several days after the appearance of NMDA-mediated currents (Dawson et al., in press). Whether there is an inducible

NOS in microglia is presently unknown, so that a role for microglia or resident macrophages in NO-mediated toxicity is unclear.

CONCLUSIONS

Realization that NADPH-diaphorase neurons are NOS-containing neurons and the discovery that NO may mediate glutamate neurotoxicity help clarify the physiological role of these neurons in neurodegenerative disorders (figure 4). Their resistance to NMDA neurotoxicity and exquisite sensitivity to kainate and quisqualate neurotoxicity have long been a puzzle. The “diaphorase” portion of NOS catalytic activity might account for their survival, because induction of NAD(P)H: (quinone-acceptor) oxidoreductase (DT diaphorase) in a neuronal cell line prevents glutamate toxicity (Murphy et al. 1991). Furthermore, these cells may possess other unique protective mechanisms against NO. Manganese-superoxide dismutase colocalizes with NADPH-diaphorase-containing neurons in the striatum (Inagaki et al. 1991). Superoxide dismutase may be partly responsible for their survival, because there is evidence that superoxide dismutase treatment of cortical cultures can attenuate NMDA neurotoxicity (Dawson et al., in press). How NO kills cells is unknown. However, NO is a highly reactive free radical, and free-radical formation has been implicated in various forms of toxicity (Choi 1990; Meldrum and Garthwaite 1990), In addition, NO can react with a superoxide anion to form peroxynitrate, which is highly reactive (Beckman et al. 1990). Furthermore, NO can complex with iron sulphur center of enzymes to inactivate them (Stuehr and Nathan 1989; Hibbs et al. 1988). Many of these enzymes are in the mitochondrial-electron-transport chain so that their inactivation would diminish the ability of cells to deal with oxidative stress.

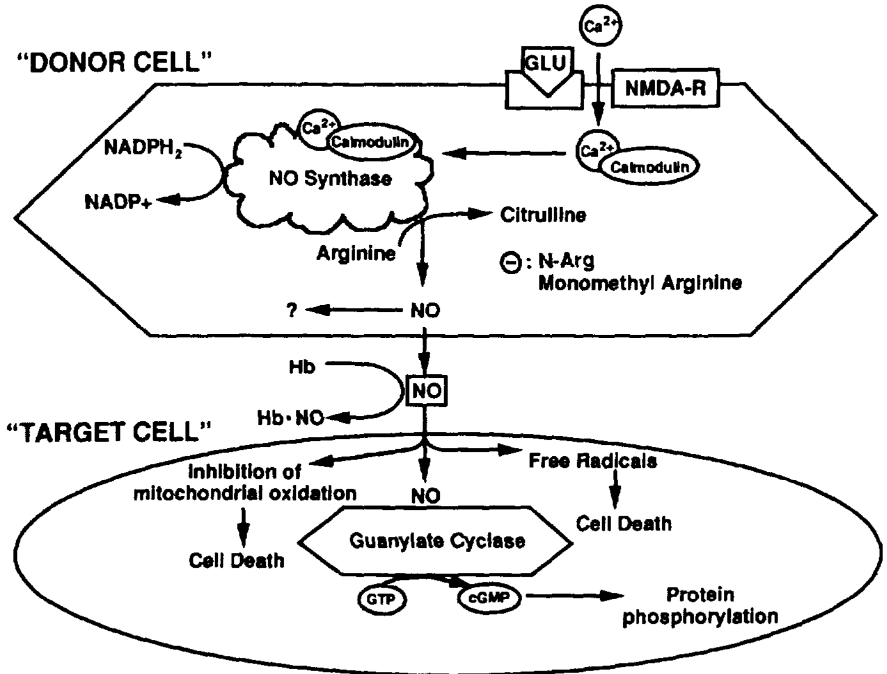


FIGURE 4. *Schematic diagram of NMDA-mediated neurotoxicity. The "donor cell" (NOS/NADPH-diaphorase-containing neurons) that activates NOS via Ca^{2+} binding to calmodulin on NMDA receptor stimulation releases NO to adjacent neurons. Under normal conditions within the target neuron, NO activates guanylate cyclase to form cGMP that activates cGMP-dependent cation channels and cGMP-dependent protein kinases that phosphorylates various proteins. Under conditions of sustained NMDA receptor stimulation, excessive amounts of NO are released that may kill adjacent "target" neurons. NO toxicity is probably mediated via the formation of free radicals. In addition, NO may combine with superoxide anion (O_{2-}) to form peroxynitrate (ONOO-) that spontaneously decomposes to the hydroxyl free radical (OH) and NO_2 (Sessa et al. 1990). Why NOS neurons survive is unknown, but they probably possess "factors" that detoxify NO. Potential candidates include the "diaphorase" portion of NOS as well as the enrichment of manganese-superoxide dismutase in these neurons.*

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DISCUSSION

Q: The NMDA-mediated toxicity might be mitigated by hypothermia. Did you do an analysis of the NADPH diaphorase temperature dependency?

A: You mean the temperature dependence of the enzyme?

Q: Right, the neuroprotective effects of MK801 are due to hypothermia, the protection against ischemic damage.

A: That is very conceivable, but we haven't looked at it.

COMMENT (Molliver): Your culture is temperature controlled.

A: At least in culture, the temperature is probably not important. In the in vivo setting it is possible, but I think Dr. Olney's work with the retina and the work of other investigators have shown that MK801 can protect against neurotoxicity despite all of these potential factors.

Q: You mentioned that sodium nitroprusside causes hypertension.

A: No, nitroarginine, the inhibitor of NO [nitric oxide] synthase. Actually, not the work that we have done, but other investigators' work has shown that nitric oxide is probably one of the major regulators of blood pressure. Animals treated with inhibitors of NO synthase develop severe hypertension. Thus, in vivo studies examining the effect of NO synthase inhibitors on neurotoxicity will be difficult to interpret, unless the animals are concurrently made normotensive or in the event that a specific neuronal NO synthase inhibitor is available.

COMMENT (Ramsay): I would like to make a comment on something that Dr. Dawson mentioned in passing, and that is the general toxic mechanism. You mentioned that nitric oxide inhibits mitochondrial systems in general. I think that the exquisitely specific effects of a lot of drugs of abuse and their metabolites in the brain are often overlooked because they also have general toxic effects and that mitochondria in particular are very sensitive to these toxic effects. Mitochondria are essential for energy, not only for the regular maintenance of the cells, but also for the defense mechanisms against radicals. That means energy. When you compromise the energy, you also compromise these mechanisms. Of the mitochondrial respiratory chain, the most sensitive step is in Complex I, which is a very complex enzyme with 23 peptides, with 5 iron sulfur clusters, and so is probably a prime target for the nitric oxide. We also know quite a lot about the effects of inhibition of Complex I from the work on MPTP [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine] and the ultimate toxin, MPP+. You can titrate Complex I with MPP+ and see immediate effects in the respiratory chain of the mitochondria. The second mitochondrial target that is important in metabolites of the neuroactive compounds is Complex III, which, though not rate limiting, is very sensitive to quinones; and quinones are very often produced as metabolites by the P450 system. A good example of this in general mechanisms of toxicity is that of acetaminophen metabolites, which cause hepatoxicity. There the quinones have been shown to interact directly with Complex III and inhibit the passage of electrons, thereby impairing the energy metabolism of the cell to such an extent that even the lactate-producing enzymes in the liver cannot support the cell. So I think that this is something to keep in mind when dealing with complex metabolites in the brain. These effects are easy to measure. You can measure them in isolated mitochondria. You can add the brain homogenates in which you are looking for metabolites. You can add them back to a mitochondria preparation. They can also be measured in cell cultures, which is rather nice. Sanchez-Ramos did that with MPP+ and showed that the heme groups of the later respiratory chain complexes stayed oxidized and couldn't be reduced, thereby showing that the block of MPP+ was in the early stages of the respiratory chain. I think that it is something to keep in mind when looking at toxicity in general.

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