Short-term Toxicity Tests for Non-genotoxic Effects
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CHAPTER 13

Short-term Tests for Neurotoxicity

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13.1 INTRODUCTION

A neurotoxic event may be defined as any change in the morphology, biochemistry or function of the nervous system, peripheral or central. Psychotropic drugs are neurotoxic chemicals according to this definition because, although they are intended to relieve mental illness, they certainly alter biochemical and functional activities of the central nervous system and, at high doses, even the morphology. However, this definition of a neurotoxic event, though broad, includes chemical contaminants in the environment.

Although it is outside the scope of this review to discuss the general problems of neurotoxicity, it should be recalled that in an evaluation of neurotoxicity, at least four aspects must be considered separately: the neurotoxic *potential*, which is a substance's ability to cause neurotoxic effects; the neurotoxic *potency*, which includes concepts of dose-range and duration of the neurotoxic effect; the neurotoxic *hazard* which is the specificity of a chemical to cause neurotoxic effects in relation to other toxic actions; and the neurotoxic *risk* which is the likelihood that a given neurotoxic effect will be produced in real life.

This review is restricted to the various ways of detecting the neurotoxic potential of chemicals that affect the central nervous system. Short-term tests will be taken to include both *in vitro* and *in vivo* tests. Frequent reference will be made to drug neurotoxicity because studies in this field are more advanced than for environmental contaminants.

13.1.1 The role of the blood/brain barrier

The brain is protected against the entry of many chemicals by the blood/brain barrier cells (Saunders, 1977). Chemicals can enter the brain by at least two mechanisms: (i) passive diffusion, which applies mostly to small lipophylic compounds and (ii) active transport of compounds of physiological importance. It is important to recognize that a chemical that is a potent neurotoxic agent *in vitro* is not necessarily neurotoxic *in vivo* if it does not enter the brain because of the blood/brain barrier.

The blood/brain barrier is not equally effective in all parts of the brain. For example, this barrier is less efficient in the so-called circumventricular organs (CVO) (Weindl, 1973). The capillaries of the CVO have large interendothelial pores (and exhibit active pinocytosis) instead of tight junctions (Brightman, 1977; Partridge, 1979). This is well demonstrated by glutamic acid, an excitatory aminoacid and a putative chemical transmitter in the brain (Shank and Aprison, 1979; Curtis, 1979; Johnston, 1979), which is neurotoxic when injected intracerebrally (Simson *et al.*, 1977; Olney *et al.*, 1975). When administered systemically, even at high doses, it does not accumulate in the brain (Garattini, 1979) although, under certain conditions, it accumulates (Perez *et al.*, 1973) in the nucleus arcuatus of the hypothalamus (Perez and Olney, 1972) where it may cause rather selective degeneration of some neurones (Olney, 1969). It has been suggested that this is probably responsible for late onset of obesity (Matsuyama, 1970) and endocrine disturbances (Lamperti and Blaha, 1976; Pizzi *et al.* 1977). This sequence of events has been best demonstrated by the use of newborn mice (Olney, 1969) receiving glutamate by parenteral injection (Garattini, 1979) at high doses capable of increasing osmolarity (Airoldi *et al.*, 1979).

It can be argued that some chemicals exert profound effects on the central nervous system without entering the brain; in fact, brain composition and/or function may change in relation to stimulation of peripheral inputs. For instance, stimulation of prolactin secretion from the hypophysis results in feed-back mechanisms on dopaminergic functions in the hypothalamus (Höhn and Wuttke, 1978; Perkins and Westfall, 1978). This mechanism has been suggested as an explanation for why domperidone, an anti-emetic drug, increases dopamine metabolites in the striatum even though it does not cross the blood/brain barrier (Ferretti *et al.*, 1983).

13.1.2 The role of metabolism

If a chemical is metabolized rapidly, very little may be available to the brain. Since, in several cases, the metabolic products are more polar and less lipophylic than the parent compound, metabolism may represent a detoxifying mechanism with respect to the brain. However, sometimes the metabolic products are still sufficiently lipophylic to cross the blood/brain barrier; in such cases, the metabolites may contribute to the neurotoxicity of the parent compound , or even be more toxic than the parent compound.

It is highly advisable to measure metabolites directly in the central nervous system. As is exemplified by the data summarized in Table 13.1, some metabolites may occur in significant concentrations in brain whereas they are present only in trace quantities (or undetectable) in the blood. Hence, analyses in readily available body fluids such as blood or urine may be unreliable indicators of the presence or absence of neurotoxic metabolites.

The capacity of nervous tissue to metabolize xenobiotics is largely unknown. In some cases, metabolic activity in the brain may result in short-lived, unstable

Table 13.1 Examples of relationship between levels of metabolites in blood and in brain as measured by the area under the curve (AUC) of cencentration–time curves of 1-aryl-piperazines after oral administration of various drugs (Caccia *et al.*, 1985)

Parent drug	Dose (mg/kg)	Metabolite	AUC (nmoles/ml × min)		Brain to plasma ratio
			Plasma	Brain	
Trazodone	25	mClPP	51	1 336	26
Etoperidone	25	mClPP	163	4 978	27
Mepiprazole	23	mClPP 27	657	24	
Enpiprazole	100	oClPP	N.D.	567	N.D.
Niaprazine	25	pFPP	681	11 442	16
Antrafenine	25	mCF_3PP	6	445	16
Millipertine	25	$oOCH_3PP$	156	841	5
Oxypertine	25	PP	210	1 920	8
Azaperone	25	Pdp	123	1 331	10
S-3608	100	TzP	N.D.	83	N.D.
Piribedil	100	PmP	N.D.	474	4
Buspirone	10	PmP	350	1 525	4

* N.D., Plasma concentrations below the sensitivity of the analytical procedure; mClPP, 1-(*m*-chlorophenyl-piperazine; oClPP, 1-(*o*-chlorophenyl-piperazine; pFPP, 1-(*p*-fluorophenyl-piperazine; mCF_3PP, 1-(*m*-trifluoromethylphenyl)--piperazine; $oOCH_3PP$, 1-(*o*methoxyphenyl)-piperazine; PP, 1-phenyl-piperazine; PdP, 1-(2-pyridyl)-piperazine; TzP, 1-(2-thiazolyl)-piperazine; PmP, 1-(2-pyrimidinyl)-piperazine.

metabolites which can covalently bind to macromolecules (proteins or nucleic acids) of the brain. Alkylation and carbomylation of some nitrosoureas which accumulate in the brain is a typical example. Another case is the neurotoxin, 6-hydroxydopamine, which enters catecholaminergic nerve terminals quite selectively by means of the uptake mechanism for dopamine and noradrenaline (Iversen, 1970) where it gives rise to unstable radicals which denature proteins (Garattini and Samanin, 1977; Heikkila and Cohen, 1971; Heikkila and Cohen, 1972).

13.1.3 Compensatory mechanisms

Within limits, the central nervous system can regenerate nerve terminals and compensate for neuronal damage. Furthermore, several nervous functions are *protected* by redundant mechanisms. Therefore, especially *in vivo*, it may be difficult to detect neuronal damage because morphological damage may not result in readily demonstrable behavioural effects.

Intracerebral administration of neurotoxins (such as 6-hydroxydopamine, 5,7-dihydroxytryptamine and kainic acid) may not elicit signs of neurotoxicity even though their ability to cause lesions in specific neurones is well documented. This makes it especially difficult to detect neurotoxicity; furthermore, there are biochemical mechanisms such as changes in turnover of certain neurochemicals or modula-

tors of receptors which compensate for neurotoxic effects. One consequence of this is the fact that major degeneration of the dopaminergic system is necessary before disturbances such as Parkinson's disease appear.

13.1.4 Difficulties in establishing short-term tests for neurotoxicity

If the definition of a neurotoxic event provided at the outset of this chapter is accepted, it is apparent that the detection of neurotoxic effects caused by certain chemicals may be extremely difficult. The brain has such a variety of cells with specialized functions, different chemical mediators and innumerable connections with other cells that it is difficult to analyse the individual and cumulative effects of a chemical on all these variables systematically. Even if it were possible to obtain neurones in culture representative of all the brain neurones (i.e. containing all the known chemical mediators), only very marginal information could be gathered because such a system will lack the network of interneuronal connections which may be important sites for toxic effects. More importantly, the functional or degenerative effects of chemicals on neurones may be age-dependent; the balance between formation and connection of axons and dendrites compared with the 'spontaneous' degeneration of neurones is known to be quite different in newborn and aged organisms. This difference in vulnerability of neurones in relation to age would hardly be seen *in vitro*.

Despite these difficulties, the field of neurotoxicity is expanding, utilizing a multidisciplinary approach. For several chemicals, there is now a body of knowledge describing the site of action and in some cases also the mechanism of action. This information is extremely important as a reference point for new chemicals which may share chemical or functional analogies.

In this chapter, the various approaches and methods to detect neurotoxic effects will be briefly described. To avoid repetition, it is stressed here that investigators must always consider a number of variables that may substantially influence the possibility of detecting neurotoxicity of chemicals including animal species, strain, sex, age, diet, concomitant pathology and interactions with other chemicals present in the environment.

13.2 NERVOUS SYSTEM CULTURE TYPES

Rapid advances in the use of cell culture systems to detect mutagenesis and carcinogenesis has aroused interest in the possibility of using more or less complex elements of nervous tissue *in vitro* as targets for neurotoxicological studies. The necessary techniques have been available for some time and have received fresh impetus from modern knowledge of cell biology. Some of these techniques have been recently carefully described by Yonezawa *et al.* (1980).

Schrier (1982) described six types of nervous system cultures for toxicologic testing; the advantages and disadvantages of each type are shown in Table 13.2.

Table 13.2 Nervous system culture types used for toxicologic testing (Schrier, 1982)

Culture type	Advantages	Disadvantages
Whole embryo	(a) Fetal metabolism probably intact but isolated from maternal metabolites (b) Normal or near-normal development and cellular and tissue interactions (c) Cultures may be initiated at a very early fetal age and toxicity tests done at any time in culture development; therefore, a wide range of development and differentiation is available	(a) Difficult to visualise toxicity at cellular and molecular levels (b) Complexity of presence of other organs and tissues (c) Reproducibility for biochemistry
Whole organ (e.g. DRG, SCG, etc.)	(a) Most neurone–neurone, neurone–glia, and glia–glia spatial relationships maintained (b) Cultures may be initiated from more mature (e.g. newborn or older) animals. Animal may be treated with potential toxins in advance or organ removal	(a) Some difficulties with hypoxia, cell death at centre of organs (b) Complex and poorly visualized intercellular interactions and communications
Explant (organo-typic cultures)	(a) Most cytoarchitecture maintained (b) Usually some flattening to allow morphologic evaluation (c) Myelination occurs reliably and is readily detected by light microscopy (d) Extracellular recording of 'organotypic' electrical activity is possible	(a) Poor morphology in living state (b) Poor visualization of individual cells and accessibility to them for intracellular recording (c) Suboptimal reproducibility for biochemistry

Table 13.2 *(continued)*

Reaggregation (of primary dispersed cell cultures)	(a) Glial multiplication limited (see also dispersed cell cultures) (b) Three-dimensional cytoarchitecture partially restored (c) Reproducibility of biochemical parameters probably better than explants (d) Maturation more complete than dispersal cell cultures	(a) Embryonic timing and dissociation procedures very crucial (b) Completely normal cytoarchitecture never restored, and degree of restoration varies among aggregates (c) Poor accessibility for electrophysiology
Dispersed cell cultures (a) Primary dispersed (b) Outgrown from explants (c) Secondary dispersed (cells obtained from primary dispersed)	(a) Increased reproducibility for biochemical studies (b) Good visualization with phase or Nomarski optics (c) Some neurohistochemical characters maintained (d) Capacity to use intracellular microelectrodes under direct microscopic observation (e) Analysis of individual synapsis possible (f) Electrophysiology, morphology and biochemistry may be done on single identified cells.	(a) Two-dimensional rather than three (b) Little or no myelination (c) Most normal cytoarchitecture lost (d) Some cell types may not survive
Cell lines (neuroblastomas, Schwannomas, hybrid lines, phaeochromocytomas, gliomas)	(a) Can obtain large quantities of single cell types for biochemical studies (b) Usually good single cell visualization (c) Ease of electrophysiologic studies (d) Provide both undifferentiated and 'differentiated' cells	(a) Near-total loss of normal cytoarchitecture (b) Cell lines may not accurately represent their counterparts *in vivo*

These six types are amenable to all possible kinds of observations—morphological, electrophysiological, biochemical, etc. The use of nervous tissue from different species including man (Buravlev, 1978) means that *in vitro* and *in vivo* data can be compared. Several cell lines are neoplastic cells such as neuroblastomas (Walum and Peterson, 1983); this may complicate the evaluation of neurotoxicity because a chemical that is cytotoxic for a neuroblastoma may not necessarily be neurotoxic for normal cells.

Although it would be impractical to summarize all studies that have been carried out with various chemicals, particularly significant results have been obtained in studies of the effects of heavy metals such as mercury (Saida, 1984; Kim, 1971), thallium (Hendelman, 1969; Spencer *et al.*, 1973) and tellurium (Lampert *et al.*, 1970). Obviously, these are areas with the best potential for correlation between *in vitro* and *in vivo* effects due to the lack of metabolism for these neurotoxic agents. It is more difficult to establish a correlation with organic compounds which undergo complex metabolic processes and have relatively selective effects. For instance, the tardive dyskinesia arising after repeated treatment with chlorpromazine or other phenothiazines seems difficult to reconcile with the morphological changes in lysosomes observed *in vitro* (Brosnan *et al.*, 1970). In order to simulate *in vivo* metabolic processes, techniques have been developed recently in which neuroblastoma cells are co-cultured with liver cells. Using this technique, cyclophosphamide (a non-reactive alkylating agent *in vitro*) becomes cytotoxic under these experimental conditions (Ericsson and Walum, 1984). However, it should be stressed that the metabolism of liver cells is not identical *in vivo* and *in vitro*.

Despite growing interest in tissue culture techniques, there is still no basis for standardization of such tests involving the nervous system because there are no reports to date concerning quality control or validation of these techniques using a number of agents to assess false positive or false negative compounds. In a recent comprehensive review, Dewar (1983) states:

> As yet, however, there has been relatively little experience of the application of tissue culture techniques in what may be termed routine neurotoxicology. Most of the neurotoxicological studies where they have been applied have been primarily concerned with mechanisms of pathogenesis using known toxicants—a task for which these techniques are particularly suited.

13.3 ELECTROPHYSIOLOGICAL METHODS

Several techniques are available to measure the electric activity of single cells (Jacques *et al.*, 1980), isolated nerves or slices from different brain areas *in vitro*. However, these techniques have not been used systematically as testing procedures for neurotoxicants although they may be potentially useful (Fox *et al.*, 1982; Johnson, 1980).

Electrophysiological methods *in vivo* have been more widely used. An account of the results obtained with the available methods has been reported by Johnson (1980) and by Fox *et al.* (1982). Table 13.3, from the review of Dewar (1983), summarizes current uses of electrophysiological techniques in neurotoxicity. Most of these techniques have the advantage that measurement can be repeated in the same animal over a period of time so that the onset and duration of neurotoxic effects can be followed. Computerized systems have enabled electrophysiology to become more quantitative and, therefore, more suitable for evaluating effects of chemicals. However, so far, electrophysiological methods have been used mainly for studies on the mechanisms of neurotoxic action of drugs. The high cost of the equipment and skill required limit the application of electrophysiological techniques in routine neurotoxicology. Lower vertebrates, such as Xenopus or Aplisia, have been used but, because of their relatively limited neuronal network, they should be used only when there is already an indication of the putative mechanism of neurotoxicity. In this respect, it has been shown that pyrethroids have no blocking activity in the peripheral nerves of frogs or rats while they are quite active on arthroprod nerve fibres (Van den Bercken *et al.*, 1973).

13.4 BIOCHEMICAL METHODS

Knowledge of the role of chemical agents in neurotransmission makes it possible to devise important experimental conditions for neurotoxicity tests. An effect of a chemical agent on a well established molecular mechanism can be extrapolated to man with more confidence than any other kinds of functional activity. The correlation between the neurotoxic action of organophosphates and their effects on acetylcholinesterase gives rise to great hopes for the evaluation of other toxic agents. In fact, Lotti and Johnson (1978) found that the inhibitory power of organophosphates against hen brain neurotoxic esterase (NTE) correlates with the effect obtained on human post mortem brain acetylcholinesterase and with the ability to produce neuropathy *in vivo*. Unfortunately, similar examples have not been found with other classes of neurotoxins.

13.4.1 Neurochemical mapping

Descriptions of all the possible biochemical points of attack for neurotoxic agents is beyond the scope of this chapter (see Damstra and Bondy, 1982). Only some aspects of the various biochemical pathways and their influence of chemical agents in brain are considered here. However, biochemical techniques, linked with morphological studies, have been used effectively to plot a map of the brain.

Horseradish peroxidase (HRP), when injected in discrete brain areas is taken up mainly by nerve terminals and is transported throughout the neurones. The location of HRP can be determined on serial sections by various histochemical procedures

Table 13.3 Electrophysiological methods in neurotoxicity testing (Dewar, 1983)

Test	Function tested	Comments	References
Maximum motor nerve conduction velocity (MCV)	Function of large diameter motor fibres in peripheral nerve	Relatively widely used as an indication of peripheral nerve damage but relatively insensitive	Fullerton, 1966; Fullerton and Barnes, 1966
Partial antidromic block-conduction velocity of slower fibres (CVSF)	Function of slower (smaller diameter) motor fibres in peripheral nerve	At present not used to any significant extent in neurotoxicology	Seppaläinen and Hernberg, 1972
Sensory nerve conduction (SNV)	Function of sensory fibres in peripheral nerve	At present relatively under-utilized in neurotoxicology. Technique is more difficult than MCV measurement but potentially more useful	Le Quesne, 1978a, b; De Jesus *et al.*, 1978
Electromyography (EMG)	Neuromuscular function	Extensively used in clinical studies but as yet not widely used in animal studies	Goodgold and Eberstein, 1977; Mendell *et al.*, 1974
Stimulus strength duration testing (S–D curves)	Detects denervation of muscles (innervated and denervated muscles differ in their thresholds to excitation)	Little used in neurotoxicology at present	Johnson, 1980
Electroence-phalography (EEG)	Spontaneous electrical activity of the brain	Particularly useful for study of effects of sleep	Takeuchi and Hisanaga, 1977
Electroretinography (ERG)	Retinal function, susceptibility of rods and cones to toxic effects	Feasible to include ERG measurements in chronic studies in dogs	Liverani and Schaeppi, 1979
Sensory evoked potentials, average evoked potentials (AEP)	Function of different sensory modalities. The AEP indicates the neural activity of the brain involved in processing sensory input	Techniques are available for use in rodents	Niemeyer, 1979

(Llamas *et al.*, 1975). It is, therefore, possible to compare distribution of HRP in normal and treated animals, revealing damage in the morphology of the nervous tissue.

To study the proximo-distal migration of proteins, labelled aminoacids can be injected in discrete brain areas. Autoradiographic methods, coupled with the light or electron microscopy, enable a map to be plotted of where the axons from a collection of neurones terminate (Wiesel *et al.*, 1974; Pickel *et al.*, 1974; Jones and Moore, 1977). Another way to map those neurones containing monoamines (such as dopamine (DA), noradrenaline (NA) and serotonin (5HT) involves the use of procedures which result in specific fluorescence (Falck *et al.*, 1962; Lindvall and Bjorkland, 1974).

Immunochemical methods have been utilized to locate a variety of enzymes and proteins within the central nervous system. The principle consists of producing specific antibodies which are then linked to fluorescent dyes. Incubation of these complexes with brain slices results in fluorescence at sites where the enzyme or the protein binds to the specific antibodies (Bock, 1978).

Labelled 2-deoxyglucose (2-DG), injected intravenously, is taken up into the neurones by an active process with an intensity related to the rate of glucose oxidation. Autoradiographic techniques can be used to detect 2-DG-6-phosphate which is formed from the phosphorylation of 2-DG but it cannot be further metabolized (Sokoloff *et al.*, 1977). Computerized imaging procedures permit quantitation of autoradiography thus enabling a 'metabolic' map of the brain to be constructed. This technique is widely used. For instance, d-amphetamine which stimulates dopamine release in the synapses increases glucose consumption and, therefore, 2-DG uptake in brain areas rich in dopaminergic terminals such as the striatum (Wolfson and Brown, 1976). This technique has a great potential for the detection of neurotoxic effects in the brain that are related not only to blockade of glucose metabolism but also to activation or inhibition of neuronal activity related to glucose metabolism.

Protein synthesis can also be measured in brain by the incorporation of various aminoacids into the protein macro-molecules. The turnover of proteins differ widely in brain (Lajtha and Marks, 1971) and, therefore, appropriate times must be carefully selected in order to study the effect of neurotoxic agents. Acrylamide (Schotman *et al.*, 1978), methylmercury (Omata *et al.*, 1978) and carbondisulphide (Savolainen and Jarvisalo, 1977) affect protein synthesis in brain but interpretation of the results is difficult because these agents may affect protein synthesis only by impairment of factors which modulate it (hormones, diet, cofactors, transport of aminoacids, etc.).

Proteins are transported along the axons at different rates; this axoplasmic flow can now be measured by various techniques. This may provide a means for further study of agents that induce peripheral neuropathies (Bondy and Madsen, 1974; Schechter *et al.*, 1979; Starkey and Brimijoin, 1979).

13.4.2 Neurotransmitter biochemistry

The functional aspects of chemical neurotransmitters as communicators among neurones has been extensively studied in recent years. From the large amount of information now available, it is clear that all stages of the neurotransmission process may be influenced by neurotoxic agents. This is exemplified by considering the monoamines.

Precursors of monoamines (e.g. tryptophan for 5HT and tyrosine for catecholamines) must enter the brain; this occurs as a consequence of active transport across the blood/brain barrier. Transport of monoamine precursors competes with the transport of other aminoacids, for example, the transport of tryptophan depends on the concentrations of the neutral aminoacids present in the blood (Osborne, 1982). After gaining access to the neurones, precursors are involved in one or more enzymic steps that result in the synthesis of specific chemical neurotransmitters. In the case of tryptophan, it is transformed by tryptophan hydroxylase to 5-hydroxytryptophan which is then decarboxylated by a *l*-aromatic aminoacid decarboxylase to form 5HT (Osborne, 1982); tyrosine is hydroxylated (tyrosine hydroxylase) to form dopa that in turn is decarboxylated to dopamine; dopamine-beta-hydroxylase catalyses the formation of noradrenaline from dopamine (Iversen and Callingham, 1971).

Once the monamine is formed, it is stored in vesicles from which it can be released into the synaptic cleft. Many chemical agents (e.g. reserpine) affect storage (Shore and Giachetti, 1978) or induce release of the monoamine (e.g. *d*-amphetamine for catecholamines (Paton, 1979), *d*-fenfluramine for 5HT (Garattini *et al.*, 1979). Once released, the monoamine is metabolized to an inactive chemical species. In the case of 5HT, it is transformed into 5-hydroxyindole acetic acid in the presence of monoamine oxidase A (Osborne, 1982); catecholamines are also subjected to the action of *O*-methylcatechol-transferase (COMT) (Iversen and Callingham, 1971). However, the principal mechanism of inactivation of monoamine is uptake inside the nerve terminals (Fonnum *et al.*, 1980). This process requires energy, is specific for given nerve terminals and is probably modulated by endogenous inhibitors of uptake. Monoamine which is not subject to metabolism or uptake interacts with postsynaptic receptors.

Recently developed methods to measure quantitatively the density (B_{max}) of certain receptors and the affinity (K_d) of neurotransmitters for their specific receptors (Bennett, 1978) have opened up new possibilities for research in central nervous tissue. A partial list of brain receptors is given in Table 13.4. It should be emphasized that the number of known brain receptors is continuously increasing. Each of these receptors may represent a target for neurotoxic chemicals. However, an effect on receptors measured *in vitro* is no guarantee of an effect *in vivo*; once a chemical is metabolized, its metabolic products may exert a different effect on receptors from that of the parent compound. The action of metabolite(s) obviously cannot be

Table 13.4 Some drug and neurotransmitter receptor types and subtypes

Subtype	Properties
	Opiate type
μ	Morphine-selective: localized in pain modulating brain regions
μ_1	Identified by very high affinity binding of numerous opiates: blocked selectively by naloxonazine; meptazinol a specific agonist; implicated in analgesia but not respiratory depression
δ	Enkephalin-selective; localized in limbic brain regions
k	Mediates sedating, less addicting analgesia; localized to deep layers of cerebral cortex; dynorphin has high affinity; mediates rabbit vas deferens contractions
σ	Naloxone insensitive; mediates psychotomimetic opiate effects; concentrated in hippocampus
ε	β-Endorphin selective; mediates rat vas deferens contractions
Cough-suppressant	Dextromethorphan-selective; reversed stereospecificity; localized to fourth ventricle floor which regulates cough reflexes
	Calcium antagonist type
Dihydropyridine	Binding dependent on Ca^{2+} and blocked by ionic calcium anagonists; regulated allosterically by verapamil; localized to molecular layer dentate gyrus, external plexiform layer olfactory bulb
Verapamil	Inhibited by physiologic Ca^{2+} levels; linked to behavioural activation by diphenylbutylpiperidine neuroleptics, sexual and cardiac effects of the phenothiazine thioridazine, and antidiarrhoeal actions of loperamide and diphenoxalate
Dilitiazem	Can involve same site as verapamil in part; allosterically regulated by dihydropyridines

	Adenosine type
A_1	Labelled by [^{3}H]cyclohexyladenosine, [^{3}H]phenylisopropyladenosine, 2-[^{3}H]chloroadenosine; lowers adenylate cyclase; adenosine analogues potent at nanomolar concentrations; stereospecific for phenylisopropyladenosine; localized to molecular layers of hippocampus and cerebellum, medial geniculate; contained on nerve terminals of cerebellar granule cells and retinal ganglion cell projections to superior colliculus.
A_2	Stimulates adenylate cyclase; adenosine analogues potent at micromolar concentrations; little stereoselectivity for phenylisopropyl-adenosine; labelled with 5′-*N*-[^{3}H]ethylcarboxamide adenosine
	α-Adrenergic type
α_1	Postsynaptic in sympathetic system; prazosin and indoramin-selective; acts through Ca^{2+} channels; little affected by guanine nucleotides
α_2	Located on sympathetic nerve terminals to regulate norepinephrine release but also postsynaptic, especially in brain; clonidine selective agonist; yohimbine- and piperoxan-selective antagonists; lowers adenylate cyclase
	β-Adrenergic type
β_1	Epinephrine and norepinephrine equally potent agonists; practalol-selective antagonist; more in heart than in lung; regional variations in brain; neuronal localization
β_2	Epinephrine more potent than norepinephrine; terbutaline- and salbutamol-selective agonists; more in lungs than heart; few regional variations in brain; more on glia than neurons
	Muscarinic cholinergic type
M_1	Concentrated in sympathetic ganglia, corpus striatum, and stomach, pirenzipine-selective antagonist; closes K^+ channels
M_2	Concentrated in hindbrain, cerebellum and heart; regulated by gallamine and GTP; inhibits adenylate cyclase

From Snyder (1984).

Table 13.4 *(continued)*

Subtype	Properties
	GABA type
A	Muscimol-selective; postsynaptic to GABA neurones, inhibited by calcium; antagonized by convulsant bicuculline
B	Baclofen-selective; on GABA and other nerve terminals; stimulated by calcium; bicuculline-resistant
Sedative-convulsant	Labelled by convulsants [^{3}H]dihydropicrotoxinin and [^{35}S]-t-butylbicyclophosphorothionate; regulated by chloride and barbiturates; linked to benzodiazepine and $GABA_A$ receptors
	Dopamine type
D_1	Enhances adenylate cyclase; labelled by [^{3}H]thioxanthenes; absent in pituitary; present in parathyroid
D_2	Lowers adenylate cyclase; labelled by [^{3}H]butyrophenones; present in anterior pituitary; responsible for antipsychotic and extrapyramidal actions
	Serotonin (5HT) type
$5HT_1$	Labelled with [^{3}H]-5HT, which is potent at nanomolar concentrations; classical 5HT antagonists are weak; regulated by guanine nucleotides, possibly linked to adenylate cyclase; mediates contraction of dog basilar artery.
$5HT_2$	Labelled with [^{3}H]spiperone and [^{3}H]ketanserin; micromolar 5HT; less affected by guanine nucleotides; mediates behavioural '5HT syndrome', contraction of oestrous rat uterus, dog and rabbit femoral and rat caudal arteries, and rat and rabbit aorta and jugular vein

detected *in vitro*. An example is trazodone, an antidepressant agent which is transformed in the body into several metabolites, including *m*-chlorophenylpiperazine (Caccia *et al.*, 1981). While trazodone interferes with 5HT2 receptors, the metabolites interact mostly with 5HT1 (Garattini, 1983). Another case is camazepam, a benzodiazepine which interacts very weakly *in vitro* with the benzodiazepine-GABA-chloride receptor complex (IC50 = 950 nM) while its metabolite temazepam shows an IC50 = 24 nM (Garattini *et al.*, 1981).

In some cases, a neurotoxic effect observed *in vitro* may not occur *in vivo* because of problems related to the blood/brain barrier. For example, domperidone is a very active agent that interacts with postsynaptic dopaminergic receptors but does not cross the blood/brain barrier (Laduron and Leysen, 1979).

In addition to the postsynaptic receptors, the brain also contains presynaptic receptors (also known as autoreceptors). These regulate the release of neurochemical mediators from the nerve terminals (Osborne, 1982; Paton, 1979). Although the fundamental role of autoreceptors has not yet been completely elucidated, it may represent another mechanism that may prove useful for detecting neurotoxicity.

Receptors are not stable entities but are continuously modulated so that when there is excessive stimulation, receptor density decreases; when there is reduced stimulation, it increases (Creese and Sibley, 1981). Neurotoxic agents may not affect the receptors *per se* but only their modulation. Furthermore, various neurones do not work in isolation but are connected to each other so that a chemical affecting one type of neurotransmitter is likely indirectly to affect other neurotransmitters too.

There is also recent evidence suggesting that certain nerve terminals contain more than one neurotransmitter (cotransmitters) (Lundberg and Hökfelt, 1983). The neurotransmitters and these interactions represent for the moment a very difficult area of research because of the extreme intricacy of these interrelations. For example, it can be shown that some tricyclic antidepressant agents which, when given in single doses, do not affect the dopaminergic system, elicit marked functional changes of this monoamine after a seven-day treatment (Borsini *et al.*, 1985a,b). These effects can be interpreted as a consequence of changes induced on other chemical neurotransmitters which, in turn, interact with the dopaminergic system.

As an example of how these various biochemical tests can be used in neurotoxicity studies, Table 13.5 summarizes the various effects of lead on neurochemical monoamines (for a review see Winder and Kitchen, 1984). It must be stressed, however, that the significance of these results in the interpretation of the overall toxicity of lead is still doubtful. The concentrations of lead reported to cause effects, especially *in vitro*, are usually higher than those which can be reached *in vivo* (Winder and Kitchen, 1984).

13.4.3 Behavioural effects

Interactions between the various events that occur in the central and peripheral nervous systems are integrated and expressed as behaviour. Behavioural

Table 13.5 Some effects of lead on monoaminergic systems in the CNS

Parameter	Effect	References
Synthesis		
Tyrosine to dopamine	Increase	Wince *et al.*, 1976
Tyrosine hydroxylase	None	Deskin *et al.*, 1980
Uptake		
Dopamine (*in vitro*)	Decrease	Silbergeld, 1977
Dopamine (*in vivo*)	None	Wince *et al.*, 1980
Serotonin (*in vitro*)	None	Silbergeld and Goldberg, 1975
Release		
Dopamine (*in vitro*)	Increase	Bondy *et al.*, 1979
	None	Komulainen and Tuomisto, 1981
Receptors		
Dopamine postsynaptic receptors	Decrease	Wince *et al.*, 1976
Dopamine D_2 (striatum)	Increase	Lucchi *et al.*, 1981
Brain levels		
Noradrenaline	Increase	Dubas and Hrdina, 1978; Jason and Kellogg, 1977
	None	Grant *et al.*, 1976
Dopamine	Decrease	Dubas and Hrdina, 1978; Jason and Kellog, 1977
	None	Golter and Michaelson, 1975
Tyrosine	None	Schumann, 1977
Homovanillic acid	Increase	Silbergeld and Chisholm, 1976
	Decrease	Govoni *et al.*, 1978
Dihydroxyphenyl acetic acid	Decrease	Govoni *et al.*, 1978
Vanilmandelic acid	Increase	Silbergeld and Chisholm, 1976
Serotonin	Decrease	Dubas and Hrdina, 1978
	Increase	Weinreisch *et al.*, 1977
5-hydroxyindolacetic acid	Decrease	Dubas *et al.*, 1978

This is only a partial list of the effects observed by studying lead neurotoxic activity. For a complete analysis, see Winder and Kitchen (1984).

disturbances are, therefore, very important indicators of neurotoxicity. Irwin (1964) developed quantitative methods to monitor many aspects of behaviour in various animal species. A number of tests which can be used for screening effects on behaviour are summarized in Table 13.6; and other tests which could be utilized as second or third order behavioural tests to obtain more detailed information are summarized in Table 13.7.

Although some of these tests require large and expensive laboratory facilities,

Table 13.6 Examples of functional and behavioural tests suitable for screening (Dewar, 1983)

Function	Test	Reference
General	Structured clinical observation (e.g. Irwin screen)	Irwin, 1964
Motor		
Spontaneous activity	Activity in activity monitor. Open field test. Exploratory behaviour	Tilson and Cabe, 1979; Finger, 1972; Delini Stula *et al.*, 1979
Impairment of motor co-ordination	Inclined plane test	Graham *et al.*, 1957
	Rotarod test. Electrorod test	Kaplan and Murphy, 1972
	Narrowing bridge test	Dewar, 1980
Muscular weakness	Forelimb grip	Boissier and Simon, 1960
	Hindlimb exterior	Cabe and Tilson, 1978; Tilson *et al.*, 1980
Fatigability	Swimming endurance	Bhagat and Wheeler, 1973
Sensory		
General responsiveness	Startle response, e.g. to puff of air	Tilson and Cabe, 1979

Table 13.6 *(continued)*

Visual	Optokinetic drum	Wallman, 1975
	Evaluation of ocular reflexes: light reflex, menace reflex and corneal reflex	Conquet *et al.*, 1979
	Localization and orientation	Marshall, 1975
	Visual cliff test	Sloane *et al.*, 1978
Olfactory	Orientation in response to odour	Marshall, 1975
Auditory	Startle response	Barlow *et al.*, 1978
	Measurement of threshold sound intensity for Preyer's reflex	Baird and Carter, 1979
Somatosensory/pain/ temperature sensitivity	Tail flick test, hot plate test, Minnesota Thermal disks	Baird and Carter, 1979; Janssen *et al.*, 1963
Orientation in space	Negative geotaxis	Fox, 1965
Physiological behaviour/ Thermoregulation	Measurement of core body temperature	Simonds and Uretsky, 1970
	Measurement of ingestion of food and water	Peters *et al.*, 1979
Learning and memory	One way avoidance task	Clark, 1966

Table 13.7 Examples of second-order and third-order behavioural tests (Dewar, 1983)

Function	Test	Reference
Motor		
Fine motor control	Operant response force (conditioning of rats to press a lever attached to a force transducer with a designated force for a given period of time)	Falk, 1970
Sensory		
Sensory deficit	Maze discriminating tests using sensory cues, e.g., T-maze test using visual discrimination	Zenick *et al.*, 1978
Visual, auditory, olfactory	Psychophysical studies—operant responding	Chiba and Ando, 1976
Somatosensory	Vibration sensitivity assessment	Maurissen and Weiss, 1980
Gustatory	Taste discrimination (using quinine)	Kodama *et al.*, 1978
Learning memory	Two-way avoidance Discriminative Y-maze Multiple T water maze	Sobotka *et al.*, 1975 Vorhees, 1974 Vorhees *et al.*, 1978

Table 13.7 *(continued)*

Affective—emotional CNS excitability	Electrical self-stimulation of the brain	Annau, 1978; Ornstein, 1979
Dissociation from environment	Conditioned avoidance response	Corsico, 1979
Physiological/ Thermoregulation	Study of circadian rhythms	Stephan and Nunez, 1977
Specific tests for: Monoamine oxidase inhibitory activity	Potentiation of head twitches by 5-hydroxy-tryptophan and reserpine reversal	Corne *et al.*, 1963; Chessin *et al.*, 1957
Induction of anaesthesia	Potentiation of 2-methoxy-4-allylphenoxy acetic acid diethylamide-induced anaesthesia	Corsico, 1979
Extrapyramidal effects (e.g. side-effects of neuroleptics)	Catalepsy test Test for dopamine receptor hypersensitivity using apomorphine-induced stereotyped behaviour	Bürki, 1979 Worms and Lloyd, 1979
Drug dependence liability	Single dose suppression test (Primates)	Swain, 1979

many others are relatively simple tests. Nevertheless, they call for experienced scientists. An extensive review on the subject has been recently published by Norton (1982) and by Tilson and Harry (1982).

13.5 CONCLUSIONS

It would be naive to believe that any single test could serve to reveal all neurotoxic effects. Even a battery of tests cannot guarantee a complete investigation covering all possible kinds of neurotoxic effects.

At the moment, there are no magic answers in the field of neurotoxicity testing. Each chemical must be studied individually; detection of its possible neurotoxic effects should be regarded not as a routine procedure but as a research project. Chemical and functional analogies may help in establishing the best procedure to be utilized but attention should always be given to unexpected neurotoxic effects.

The importance of *in vitro* tests cannot be overstated; at the present time, *in vitro* tests, although not a complete replacement for *in vivo* experiments, appear to be a complementary tool for understanding mechanisms of action. The specialization of the nervous system is such that it seems impossible to obtain cell lines or organ cultures representative of all these functions for use *in vitro*. Nevertheless, efforts must continue to develop culture conditions for nerve cells that avoid loss of their biochemical characteristics.

In vivo tests also have limitations because all neurotoxic effects are not expressed in detectable symptoms and because compensatory mechanisms operate in the brain. *In vitro* studies following *in vivo* treatment (*ex vivo*) may partially obviate this difficulty. The pressure of some groups to reduce *in vivo* tests because they are not predictive of human neurotoxicity may be understandable, but it is difficult to understand how *in vitro* tests could be more predictive when they obviously suffer from excessive simplification.

Neurotoxic effects in fetuses, newborn and aged organisms represent important aspects of neurotoxicology that deserve special attention. There is also a need to understand better which neurotoxic effects occur in man because of exposure to chemical contaminants. In this respect, epidemiological studies should be encouraged because the results could help in planning more meaningful experimental testing.

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