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The Biochemical Evaluation of Neurotoxic Damage

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The Biochemical Evaluation of Neurotoxic Damage. BONDY, S. C. (1986). *Fundam. Appl. Toxicol.* 6, 208-216. The specific problems related to the study of chemicals that damage the nervous system are discussed. Such damage may be direct or may occur by a series of sequential events. Such a series of events may involve other organs and biochemical targets not confined to nerve tissue. The complexity of investigation of the mode of action of rather nonspecific toxic agents requires that a circumscribed objective be defined and examples of such goals are given. One of these is to attempt to determine which neuronal circuits are damaged by a given toxic agent and how this might cause alterations of behavior. Acrylamide and triethyl lead are used to exemplify the application of neurotransmitter receptor analysis to correlation of deranged nerve activity patterns with modulation of behavior. Such evaluation can set the stage for more detailed biochemical studies of neurotoxic mechanisms. © 1986 Society of Toxicology.

Neurotoxic damage can be caused by many pharmacological agents and biological neurotoxins. These types of compounds have an inherent man-made or evolutionary design and are generally harmful in rather selective ways. Neurotoxic injury can also result from nonspecific, environmentally prevalent chemicals. These may be of natural origin (for example various metals or minerals) or may be products of industrial processes. Unlike pharmacological agents and natural toxins such as snake venoms, many nonspecific toxic agents are industrial products not designed to affect biological function. They are therefore likely to exert their effects by interacting with more than a single biological site. This potential multiplicity makes it difficult to identify the precise mechanisms by which damage to the organism is effected. For this reason, sites of action of many toxic agents are generally less well understood than those of pharmaceutical agents. Of course, some environmentally prevalent neurotoxic compounds such as pesticides were specifically developed for their

effects on the nervous system of insects and thus may have rather precise targets.

Altered behavior is an end point of the effects of all toxic agents but not all such materials are classified as neurotoxicants. Neurotoxicity is generally understood to imply a direct effect on nerve tissue. However, most environmental agents that appear to primarily cause damage to the nervous system are also harmful to other tissues and organs. In view of the especial vulnerability of the adult brain to anoxia, many chemicals interfering with normal oxidative metabolism, such as cyanide ions or carbon monoxide, can under certain circumstances appear selectively neurotoxic.

GOALS OF NEUROTOXIC STUDY

Biochemical study of neurotoxicity can have several goals and each objective requires a differing research strategy.

A. *The detection of initial sites of impact of a neurotoxic agent.* This goal is of value in furthering understanding of the direct mechanisms involved in the initiation of toxicity. Such work may be oriented toward the more ubiquitous types of cell component. For ex-

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ample, membrane integrity can be assessed by a variety of means ranging from measurement of membrane fluidity to study of the integrity of the blood brain barrier. General metabolic events such as rates of oxidative phosphorylation and macromolecule synthesis may also find neurological expression, when altered throughout an animal. One feature of the approach of identifying the initial sites of action of xenobiotics is that the opportunity exists of reproducing such effects *in vitro*. This allows interactions to be studied under more defined conditions and strengthens the argument that a primary event is involved. Thus, lead will inhibit δ -amino levulinic acid dehydratase *in vivo* and *in vitro* (Moore *et al.*, 1980), suggesting a direct action on the heme biosynthetic pathway.

Direct sites of action of neurotoxic chemicals may not always be within the nervous system. Many chemicals are metabolized by enzymes to form toxic materials. The liver is especially capable of both detoxification and "toxification" reactions. Tetraethyl- and tetramethyl-lead compounds and acrylamide appear to be hepatically metabolized to more neurotoxic materials than they themselves are (Grandjean and Neilsen, 1979; Agrawal *et al.*, 1981).

The major brain-body interactions are funnelled through the hypothalamus and small changes here can give rise to a cascading effect and thus cause much larger somatic changes (Fig. 1). The magnification factor in the sequence between release factors $\rightarrow$ trophic hormones $\rightarrow$ hormones $\rightarrow$ target tissues, may be around 1000-fold for each step. Thus any modulation of hypothalamic metabolism may have disproportionately large indirect consequences. Damage to peripheral glands such as testis or adrenals can also upset the normal hypothalamohypophyseal feedback mechanisms by which their secretion is regulated. For example, while manganese is generally thought to directly damage certain nerve pathways, it also has a damaging effect on the testis (Imam and Chandra, 1975). The resulting reduction in testosterone levels (Laskey *et*

FIG. 1. The neuroendocrine axis; key role of the hypothalamus.

al., 1983; Hong *et al.*, 1984) may also modify an animal's behavioral characteristics. Altered production of trophic hormones can then cause a variety of behavioral changes. Many trophic hormones such as prolactin and ACTH affect the nervous system directly in addition to their effect on somatic glands (Van Ree *et al.*, 1978). The brain-body interaction is sufficiently complex that the distinction between a "toxic" and a "neurotoxic" agent is rather arbitrary.

B. The detection of derangement of specialized features of the nervous system. This approach emphasizes the damage caused to chemicals or processes that are either unique to, or preponderant in, nerve tissue. Chemicals that are confined to the nervous system include the protein and lipid components of myelin. The process of myelination is susceptible to a variety of toxic agents. These may act by apparently directly damaging myelin (e.g., triethyl tin) or may exert an adverse effect on general development and thus indirectly retard myelin deposition. Inorganic lead may partially act in this latter manner (Michaelson, 1980). Another way in which myelin development can be indirectly affected is by way of neuronal damage, since axonal diameter plays a role in determining the extent of oligodendroglial production of myelin (Friede, 1972). Myelination may even be affected by the extent of electrical activity in the ensheathed nerve (Kingsley *et al.*, 1970).

Axoplasmic transport is a process that represents an exaggeration of a property found in all cells. This enhanced emphasis on the movement of proteins and other materials, is associated with an unusually high content of microtubule protein in nerve tissue in the form of neurotubules. This makes the nervous system sensitive to certain kinds of antimitotic agent such as colchicine and vinblastine although the rate of mitosis in adult nerve tissue is very low. Thus these agents can have behavioral effects that are not related to their potent antimitotic activity (Clingbine, 1977). Other agents such as *n*-hexane, methylbutylketone, and aluminum interfere with axoplasmic transport but have no antimitotic properties. The agents probably damage the neu-

rofilaments (Shelanski and Liem, 1979). Other vulnerable features of axoplasmic transport include its dependence on the calcium ion which can be competed with by certain heavy metals (Hammerschlag *et al.*, 1977). The extent of axoplasmic transport can be assayed in central nervous tissue by utilization of the visual system (Reichert and Abou-Donia, 1980). The avian visual system has a prominent retina and a totally decussated optic chiasm (Fig. 2). This allows quantitation of the extent to which protein synthesized in the retinal ganglion cell body, can be "exported" to the axonal terminus (Bondy and Madsen, 1974). For example, a single systemic dose of tri-*ortho*-cresyl phosphate (TOCP), given to hens, results in a diminution of slow axoplas-

FIG. 2. Radioautography of chick visual pathway 6 days after injection of 500 μCi [^3H]proline into the left eye.

TABLE 1

PERCENTAGE OF RETINALLY SYNTHESIZED PROTEIN TRANSPORTED TO THE DISTAL TERMINUS
OF THE RETINAL GANGLION CELLS IN TOCP-TREATED HENS

Time of exposure prior to kill	Fast (5 hr) transport		Slow (10 days) transport	
	Control	TOCP	Control	TOCP
11 days	1.91 $\pm$ 0.23	1.82 $\pm$ 0.23	12.3 $\pm$ 2.3	11.4 $\pm$ 2.2
24 days	1.76 $\pm$ 0.12	1.57 $\pm$ 0.30	12.6 $\pm$ 0.7	8.4* $\pm$ 0.3

Note. TOCP dose was 75 mg/kg body wt. 10 μ Ci [3 H]proline were injected monocularly 5 hr or 10 days before assay of labeled retinal and tectal protein. The proportion of labeled protein transported was calculated using the formula: (Label in optic tectum)/[(Label in retina) + (label in optic tectum)]. A correction was made for protein in the tectum labeled by blood-borne isotope by determining radioactive protein in the optic lobe ipsilateral to, and not innervated by, the injected eye.

* Differs from control ($p < 0.05$).

mic transport, 24 days after administration of this neurotoxic organophosphate (Lapadula *et al.*, in preparation, Table 1). Any interference with this process can have severe transynaptic consequences in view of the trophic role of many substances secreted at the synapse (Schwab and Thoenen, 1977).

C. The correlation of behavioral changes caused by toxic agents, with altered rates of nerve transmission. This type of study attempts to account for the altered behavior of animals treated with a toxic agent, in terms of deranged underlying neuronal circuitry. This involves the measurement of chemicals or phenomena that are confined to distinct morphological and anatomical components of nerve tissue. Such changes may be indirect and at the terminus of a series of secondary effects of the toxic agent (Fig. 3).

Behavioral changes caused by neurotoxic agents include tremor, convulsions, paralysis, and altered states of arousal or mentation.

These are rather nonspecific and each could be caused by a variety of neuronal changes. A major area of our interest has been the relation between altered behavior and modulation of defined nerve pathways by toxic agents. This approach does not attempt to directly identify primary molecular mechanisms. Delineation of damaged neuronal species can, however, give clues to the series of steps that lead to altered behavior.

THE NEUROTRANSMITTER RECEPTOR APPROACH

The basis of receptor studies is the frequently reported inverse relation between the activity level of a nerve pathway and the neurotransmitter binding capacity of the postsynaptic receptors. Such a generalization has exceptions and is further complicated by the existence of presynaptic receptors which may not exhibit such a response. However, the concept

FIG. 3. Possible sites of attack by neurotoxic agents.

has been successfully applied in many areas of neuropharmacology. Preliminary studies were performed using some neurotoxic agents already known to possess a degree of specificity. Tri-*ortho*-cresyl phosphate is known to have an inhibitory effect on acetylcholinesterase and thus cause cholinergic hyperactivity (Davis and Richardson, 1980; Abou-Donia, 1981). Avian species are especially vulnerable to this industrial product and we have assayed receptor binding intensity in the forebrains of TOCP-treated hens (Ali *et al.*, 1984). The muscarinic cholinergic receptor binding capacity was depressed in treated hens while no change was detected in six other receptor species (Table 2). This implied that the firing rate of the muscarinic cholinergic neurons was elevated or that the persistence of acetylcholine in the synaptic cleft was enhanced due to a reduced rate of catabolism. If less were known about TOCP, such data could lead to a closer examination as to what the cause of such cholinergic hyperactivity might be.

Another neurotoxic agent whose locus of damage is somewhat understood is manganese. This metal appears to selectively damage dopaminergic circuitry of treated animals (Donaldson *et al.*, 1961). Exposure of humans to excessive amounts of this element results

initially in a reversible, schizophrenic-like state, and later in a permanent Parkinsonian-like condition (Cook *et al.*, 1974). This suggests an initially high level of dopaminergic activity followed by death of dopamine neurons (Cotzias *et al.*, 1974). Various receptor species were examined in brain regions of rats repeatedly dosed with manganese (Seth *et al.*, 1981a). Striatal spiroperidol binding was elevated in exposed rats and this was attributed to damaged dopamine neurons. Cerebellar GABA receptors showed increased binding capacity, perhaps reflecting activation of inhibitory neurons in response to reduced dopaminergic activity. High-pressure liquid chromatography of serotonin, dopamine, and their metabolites suggested increased turnover of these monoamines in exposed rats (Hong *et al.*, 1984). The apparent contradiction between dopamine and serotonin metabolite concentrations (suggesting increased neuronal activity) and receptor binding data (suggesting decreased activity) has a parallel in haloperidol-treated rats. Under certain conditions, the dopamine receptors can be elevated in rats while behavioral tests indicate dopaminergic hyperactivity (Fuxe *et al.*, 1980).

There are several possible reasons that may account for apparent discrepancies of this nature. (1) If a proportion of a neuronal group is damaged or destroyed, the surviving neurons may compensate by becoming excessively active. Thus, levels of receptors and metabolites of neurotransmitters may not always be altered in a consonant manner. (2) The direction in which presynaptic receptor levels respond to altered rates of neuronal firing in the case of adrenergic neurons, is in a direction opposite to that of postsynaptic responses (Lee *et al.*, 1983). However, it is premature to state that this can be considered a general phenomenon. Presynaptic receptors are involved in the regulation of stimulus induced secretion of neurotransmitters by feedback inhibition. Therefore, their downregulation in a circuit with unusually low activity may increase the presynaptic release rate of transmitters within the synaptic cleft and thus potentiates postsynaptic

TABLE 2

HIGH-AFFINITY BINDING IN FOREBRAINS OF HENS
EXPOSED TO TOCP

Ligand	Presumed receptor site	Control	TOCP
QNB	Muscarinic cholinergic	147 ± 8	116 ± 4*
Muscimol	GABA	21 ± 4	23 ± 2
Spiroperidol	Dopamine	65 ± 6	72 ± 8
Diazepam	Benzodiazepine	14 ± 1	11 ± 1
Dihydro- alprenolol	β-Adrenergic	15 ± 3	13 ± 2
Strychnine	Glycine	55 ± 7	57 ± 4
Serotonin	Serotonin	35 ± 3	32 ± 5

Note. Binding expressed as pmol/g protein ± SE.

* Differs from control ($p < 0.05$).

effects. Thus, the regulation of both pre- and postsynaptic receptors is in a direction tending to maintain homeostasis. (3) Many biological responses are multiphasic and overlapping. Thus patients chronically treated with neuroleptics may simultaneously present tardive dyskinesia and stereotypy (implying dopaminergic hyperactivity) and Parkinsonian symptoms (implying abnormally reduced activity of dopamine neurons) (Barnes *et al.*, 1980). These complications make the transmitter species affected by a toxic agent more readily detected than the directionality of changes in firing rate.

A widely used chemical that is damaging to peripheral nerve tissue is acrylamide. This chemical had been found to cause behavioral changes that also suggested involvement of the central nervous system (Schaumberg and Spencer, 1978). There was, however, no clear indication as to which transmitter system might be modified by acrylamide. For this reason, a broad survey of receptor status in acrylamide-treated rats was carried out (Bondy *et al.*, 1981; Agrawal *et al.*, 1981). At the lowest acrylamide doses used, striatal dopamine receptor binding was elevated suggesting selective damage to, or suppression of, dopamine neurons (Table 3). At higher doses or repeated doses of acrylamide, an increasing number of receptor species appeared to be elevated. This may have been due to a broadening involvement of, and damage to, other nerve pathways.

Further work showed that the elevation in dopamine receptors was (a) reversible, (b) attributable to post- rather than presynaptic receptors (Hong *et al.*, 1982), (c) blocked by pretreatment of rats with SKF 525a, a mixed function oxidase inhibitor (Agrawal *et al.*, 1981). This latter finding implied that observed changes in dopamine receptors were mediated by a metabolite of acrylamide. The behavioral response induced by apomorphine (increased motility, stereotypy) was suppressed in acrylamide-tested rats, further suggesting damage to the dopamine neurons of the nigrostriatal pathway. The inhibition of reinforced bar pressing by clonidine and chlordi-

TABLE 3

REGIONAL RECEPTOR BINDING IN RATS RECEIVING ACRYLAMIDE

Regions	Receptor species	Acrylamide dose (mg/kg body wt/day)									
		24-hr exposure after a single dose					10 doses over 14 days				
		0	25	50	100	0	5	10	20		
Striatum	Dopamine	334 ± 13	413 ± 11*	417 ± 11*	481 ± 32*	237 ± 15	371 ± 15*	314 ± 17*	366 ± 11*		
	Acetylcholine (muscarinic)	527 ± 26	490 ± 25	479 ± 21	549 ± 55	472 ± 22	591 ± 30*	594 ± 26*	618 ± 16		
Frontal	Benzodiazepine	76 ± 6	79 ± 6	63 ± 5	80 ± 4	43 ± 6	43 ± 5	34 ± 2	40 ± 4		
Cortex	Serotonin	66 ± 5	72 ± 5	70 ± 4	84 ± 7*	65 ± 3	76 ± 6	69 ± 6	97 ± 5*		
Cerebellum	GABA	640 ± 64	768 ± 48	576 ± 48	544 ± 32	280 ± 24	312 ± 24	280 ± 16	440 ± 24*		
Medulla	Glycine	558 ± 96	636 ± 24	654 ± 42	690 ± 35*	420 ± 18	456 ± 12	450 ± 18	492 ± 36*		

Note. Binding expressed as pmol/g protein ± SE.

* Differs from zero-dose ($p < 0.05$). Zero-dose animals received corresponding injections of water (taken from Bondy *et al.*, 1981; Agrawal *et al.*, 1981).

azepoxide was unchanged after acrylamide treatment but the corresponding apomorphine depression was exacerbated (Tilson and Squibb, 1982), suggesting a degree of specificity of dopaminergic changes. The concentrations of striatal dopamine or of its metabolite dihydroxyphenylacetic acid (DOPAC) were not detectably altered in the striatum of treated rats. Cortical dopamine and DOPAC were depressed in repeatedly dosed rats while widespread elevation of 5-hydroxyindoleacetic acid levels in acrylamide-treated animals implied serotonergic activation (Ali *et al.*, 1983). Thus, receptor assay may in some cases have the potential of being a more sensitive index of deranged circuitry, than neurotransmitter levels. Such initial data should be supplemented with measurements of neuronal activity (i.e., electrophysiological measurements or transmitter turnover studies) and where possible, behavioral or physiological measurements of the reactivity of the postsynaptic site. The receptor approach can be especially useful in pinpointing lesions caused by neurotoxicants, such as acrylamide, where little data exists to suggest the neuronal tracts affected.

A neurotoxic chemical that we are currently studying is triethyl lead chloride (TEL). We have examined the benzodiazepine binding sites at various times after a single injection of 7.9 mg/kg body wt of TEL in adult male rats. A reversible depression of the extent of hippocampal benzodiazepine binding was observed and this was regionally specific in that it was not seen in the frontal cortex or striatum. This change correlated temporally with a transient analgesia. However, the reversal of such behavioral changes by administration of chlordiazepoxide has not been clearly demonstrated. Receptor data can be most closely linked to behavioral changes by study of the behavioral effects of various pharmacological agents in animals treated with toxic materials (Walsh and Tilson, 1984).

Exposure of developing rats has been compared to similar exposure of adults using chlordecone and acrylamide as representative agents. In the adult male chlordecone de-

creases striatal spiroperidol binding while this binding is increased by acrylamide. This situation is reversed after gestational and neonatal exposure of immature male rats (Agrawal and Squibb, 1981; Seth *et al.*, 1981b). In this case acrylamide causes a depression and chlordecone, an elevation of spiroperidol binding sites (Fig. 4). This may be due to the differing response of postsynaptic receptors after adult denervation in comparison to failure of innervation in the immature animal. Damage to afferent neurons before axons reach their targets may cause failure of receptor development rather than supersensitivity in target cells (Rosengarten and Friedhoff, 1979). Conversely, a high level of activity could play an inductive role during ontogenesis rather than effecting receptor down regulation. Information gained from receptor data in developmental studies has therefore to be interpreted with this possibility in mind.

CONCLUSIONS

Longer exposure to a toxic agent potentially increases the complexity of the adaptive re-

FIG. 4. Spiroperidol binding to striatal membrane of male rats exposed to toxic agents. Dosing conditions used to obtain exposed males were as follows. Chlordecone was given in the diet (6 ppm) of dams for 60 days prior to mating throughout gestation and lactation. At 12 days of age, offspring were maintained on a normal diet before being killed at 30 days. In adult studies, dietary chlordecone was (30 ppm) given for 90 days preceding sacrifice. Acrylamide was given to dams orally (20 mg/kg body wt) daily while gestational age was 7–17 days. Offspring were killed 14 days postnatally. For adult rats, 20 mg/kg acrylamide was given daily for 10 of the 14 days preceding sacrifice.

action. Such adaptation can be extremely successful if allowed to occur over an extended period. The astonishing compensatory capacity of the young brain is exemplified by the fortuitous discovery of a case of hydrocephalus in a young man, which resulted in a final brain weight of around 10% of the normal. This man had obtained a mathematics degree and showed no obvious neurological abnormality (Lewin, 1980).

The receptor changes found in animals exposed to neurotoxic agents often represent homeostatic adaptive responses. These responses may be especially relevant to chronic exposures to lower levels of harmful agents. Acute poisoning of an animal may not allow time for such changes or may render the animal incapable of such modulating responses. The receptor-oriented type of interpretation of the action of substances harmful to the nervous system may have practical application in furthering understanding of various hazardous industrial and environmental chemicals. Knowledge concerning disruption of defined nerve circuits may also assist in the development of therapeutic strategies following exposure to neurotoxic chemicals.

A rather general disruption of membrane stability or inhibition of an ubiquitous enzyme might ultimately find expression by disturbance of a particularly vulnerable nerve pathway. This apparent selectivity does not imply that the primary locus of a toxic agent is confined to that neuronal species. For example, catecholamine neurons may be especially sensitive to oxidative conditions because of the susceptibility of several monoamines to oxidation (Balentine, 1968). An enzyme that is very readily damaged by oxygen is glutamate decarboxylase (Tunnicliff and Wood, 1974) and thus oxidizing conditions may cause an elevation of the excitatory transmitter glutamate, and depression of levels of its decarboxylated product, GABA, an inhibitory transmitter. This imbalance may lead to convulsions and brain damage. This may, in part, account for the susceptibility of the developing retina to hyperbaric concentrations of oxygen. Failure of oxygen supply may also damage

distinct areas of the brain (Daughtrey and Norton, 1982). A limitation of receptor evaluation is that this approach does not automatically throw light on the series of biochemical steps leading to these adaptive changes. Mechanisms of action at the molecular level can only be reached by working backwards toward the initial targets of a toxic agent. The "mechanism of action" of a neurotoxic agent can only be defined when all intermediate steps are delineated. In view of the multifocal assault of many toxic agents, it may be wise to define a more limited and concise research goal. It is important, however, to be aware of the complex sequence of events that lead to the expression of neurotoxicity.

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