CHAPTER 16

Toxicology of the Nervous System

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16.1 INTRODUCTION

Many substances alter the normal activity of the nervous system. Sometimes these effects are immediate and transient, like the stimulatory effect of a cup of coffee or a headache from the fresh paint in your office. Other effects can be much more insidious, like the movement disorders suffered by miners after years of chronic manganese intoxication. Many agents are safe or even therapeutic at lower doses but become neurotoxic at higher levels. Trace metals and pyridoxine (vitamin B-6) fall into this category of dose-dependent effects. Since these agents affirm the maxim, "the dose makes the poison," it becomes necessary to have a meaningful definition of nervous system poisoning, or neurotoxicity. *Neurotoxicity refers to the ability of an agent to adversely affect the structural or functional integrity of the nervous system.* Structural damage to nervous system components usually results in altered functioning, although the reverse is not always true. Alterations in nervous system function may occur through toxicant interactions with the normal signaling mechanisms of neurotransmission, resulting in little or no structural damage. Nevertheless, it is easier to identify alterations, be they structural or functional, than it is to define adversity. For example, the stimulant effect of a morning cup of coffee may be too anxiety provoking for some individuals but a necessity to others.

In this chapter a brief introduction to the nervous system is presented and its functions are described. A discussion of some of the mechanisms of structural and functional neurotoxicant effects follows. These descriptions are not exhaustive, they are meant to illustrate the concepts of toxicant interaction with the nervous system. Finally some methods for testing toxicant effects in the nervous system are explored.

16.2 THE NERVOUS SYSTEM

Most multicellular animals possess a nervous system. In every case the function of the nervous system is to receive information about the external and internal environment, integrate the information, and then coordinate a response appropriate to the

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environmental stimulus. In addition to these basic vital functions, the nervous systems of higher organisms are responsible for feeling, thinking, and learning. All of the other organ systems of the body are subject to control by the nervous system; thus damage to this "master" system by toxicants can have far-reaching and even devastating effects.

In vertebrates there are two major components of the nervous system. Although these two systems are anatomically separable, they are contiguous and they function interactively. The brain and spinal cord comprise the central nervous system (CNS), and the nervous tissue (ganglia and peripheral nerves) outside the brain and spinal cord comprise the peripheral nervous system (PNS). The PNS can be further divided into the somatic and autonomic nervous systems. Somatic afferents carry sensory information from the skin, muscle, and joints to the CNS, while motor efferents innervate skeletal muscle to cause contractive movement. The autonomic nervous system can be thought of as a motor system for visceral organs, since it projects to these organs to innervate and control the function of smooth muscle, cardiac muscle, and endocrine and exocrine glands. The autonomic nervous system is further divided anatomically and functionally into the sympathetic and parasympathetic subdivisions. Most organs are innervated by both subdivisions, and their influences generally oppose one another. For example, stimulation of sympathetic nerves increases heart rate, while stimulation of the vagus nerve, the primary parasympathetic innervation of the heart, slows its rate of contraction.

16.2.1 The Neuron

The basic unit of the nervous system is the neuron, a type of cell that is structurally and functionally specialized to receive, integrate, conduct, and transmit information. Although neurons are far more diverse than any other cell type in the body, they do have some common features. Neurons are polarized cells; that is, they have different characteristics on one end of the cell compared to the other (Figure 16.1). Typically the end of the neuron that receives information in the form of neurotransmitter stimulation from other neurons is highly branched into a region known as the dendritic tree. The branches are sometimes studded with projections, known as spines, which contain receptors that recognize and are activated by neurotransmitters. It is here that the neuron is in close contact with other neurons via specialized structures called synapses. Synapses are areas of close apposition where one neuron, the presynaptic neuron, releases neurotransmitter into the gap, or cleft, between the two neurons. The postsynaptic neuron then recognizes this chemical signal via receptors that are clustered in small densities opposing the presynaptic neuron. Once the neurotransmitter signal is recognized by its receptors, the dendritic region of the neuron transmits the information as intracellular and electrochemical signals to the regions of the neuron where signal integration takes place. In the typical neuron the arborizations of the dendritic tree converge on the soma, or cell body, where the nucleus and most of RNA- and protein-synthesizing machinery exist. The cell body usually then gives off a single axon, and it is in the region where the axon leaves the cell body (the axon hillock) that signals converge to be integrated into an all-or-none response.

Neurotransmission down an axon is in the form of electrochemical signals. In the resting state the interior of the axon is negatively charged with respect to the exterior. The membrane is then said to be polarized, and the charge difference across the membrane in this resting state is approximately -70 mV. Small depolarizing potentials

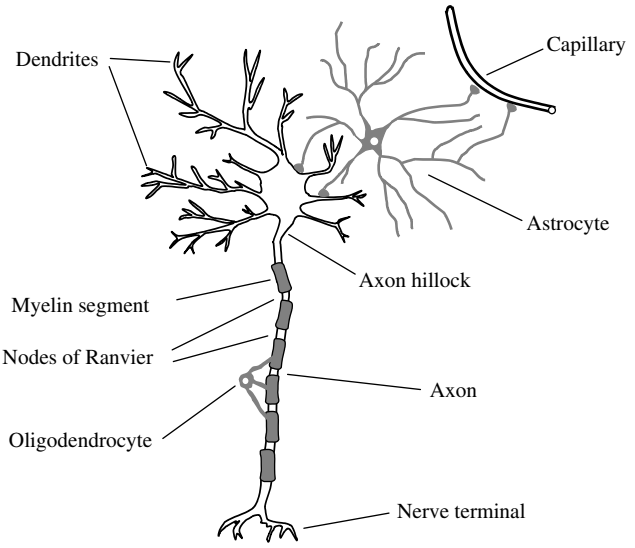

Figure 16.1 A neuron with accompanying astrocyte and myelinating oligodendrocyte.

arrive at the axon hillock from the dendritic regions where receptors have been stimulated, and this stimulation results in the opening and closing of ion channels. The depolarizing potentials occur primarily because of the opening of sodium channels, allowing sodium to transfer down its concentration gradient to the interior of the cell. Sodium brings with it a positive charge, and so the membrane in the region where the sodium channel opens becomes depolarized. The depolarization is then detected by voltage-sensing sodium channels, which allow further influx of sodium. When the spatial and temporal summation of these signals reaches a certain threshold (generally about +50 mV), the axon will generate an action potential at the axon hillock. Once this occurs, all of the sodium channels in the vicinity open, allowing a massive influx of sodium. Sodium channels stay open for only a short period of time, and once they close, they cannot reopen for a while. On the other hand, as sodium channels further down the axon sense the voltage change, they open, and thus a feed-forward effect is created. The membrane is repolarized by the opening of potassium channels, which respond in a slightly delayed fashion, to the same signals that stimulate the sodium channels. As the potassium channels open, potassium rushes out of the cell down its own concentration gradient. This, combined with the closing of the sodium channels, produces a net efflux of positive charge, thereby repolarizing the membrane. The process of depolarization/repolarization continues down the length of the axon. In myelinated axons, the ion channels are clustered in regions between the segments of myelin in regions known as nodes of Ranvier. The myelin segments serve to insulate the axon, and they allow the action potential to jump from one node to the next, in a process called saltatory conduction (Figure 16.2). This results in much faster propagation of the action potential down the length of the axon.

Axons terminate at neuromuscular junctions, in effector organs (e.g., the heart), and in synapses with other neurons. When the action potential reaches the terminal of the axon, the depolarizing impulse stimulates the release of neurotransmitter from the terminal into the cleft between the presynaptic membrane and its effector or receiving

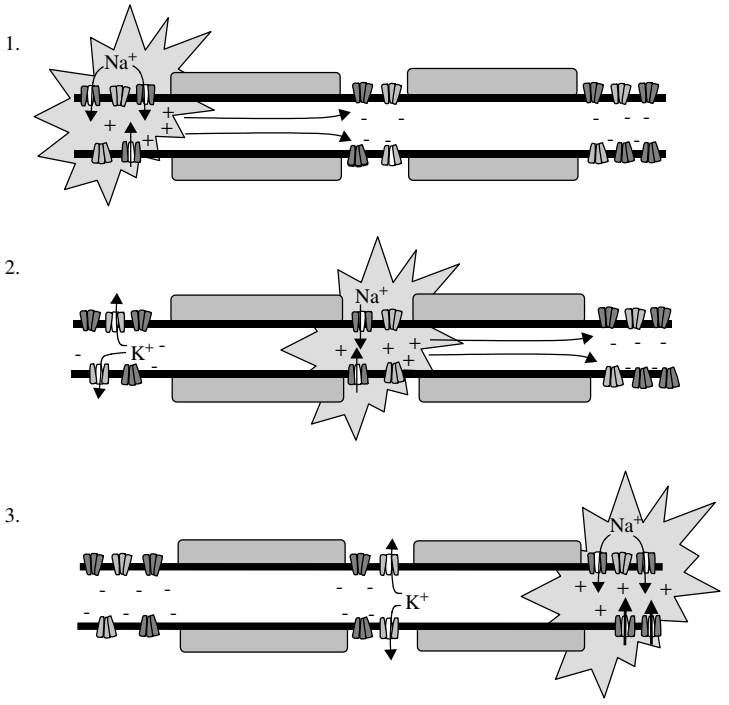

Figure 16.2 Saltatory conduction. Myelin acts as an insulator to prevent current loss as the action potential travels down the axon. Sodium and potassium channels are clustered at the Nodes of Ranvier, where there is no myelin. Action potentials jump from one node to the next, reducing the overall membrane area involved in conduction, and speeding up electrical transmission.

neuron. The process of release usually involves the presence of packets of neurotransmitter called synaptic vesicles (Figure 16.3). These vesicles dock at the presynaptic membrane and, when stimulated to do so, fuse with the membrane to release their contents into the synaptic cleft. The signal to fuse is thought to be primarily an influx of calcium, mediated by calcium channels on the presynaptic membrane that are sensitive to changes in voltage. Proteins on the vesicle membrane and the presynaptic membrane form complexes with one another, and when stimulated by the localized increase in calcium ion concentration, mediate the fusion of the two membranes and release of neurotransmitter. Electrical signals at work within the neuron are thus converted to chemical signals at work between neurons in the form of neurotransmitters.

16.2.2 Neurotransmitters and their Receptors

Neurotransmitters are recognized by receiving neurons, neuromuscular junctions, or end effector organs via receptors that lie on the postsynaptic membrane. Receptors are generally selective for the neurotransmitter that they bind. The type of signaling that is characteristic of a given neurotransmitter is usually the result of the form of receptor to which it binds. For example, some receptors, like the nicotinic acetylcholine receptor found in neuromuscular junctions, are ion channels. The stimulation of the nicotinic

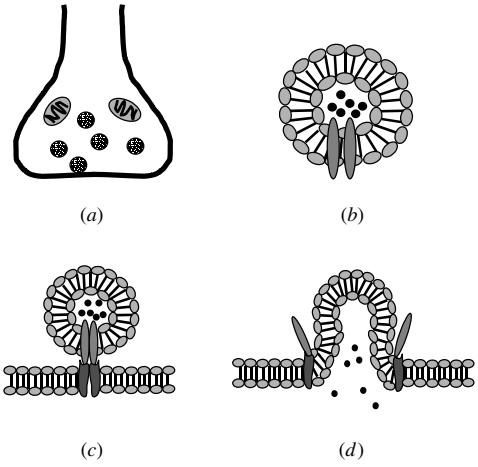

Figure 16.3 Neurotransmitter release. (*a*) Presynaptic nerve terminal containing vesicles and other organelles. (*b*) Neurotransmitter-containing vesicles are made of lipid bilayers. Associated proteins participate in the release process. (*c*) The vesicle associates with the presynaptic membrane via protein complexes that mediate release. (*d*) Release of neurotransmitter into the synapse is by protein-mediated fusion of vesicle and presynaptic membranes.

receptor by acetylcholine results in the opening of its channel, which is permeable to sodium. The influx of sodium then serves to depolarize the muscle membrane that receives acetylcholinergic innervation. Neurotransmitter receptors that are ion channels thus mediate very fast and short-lived neurotransmission, particularly when compared to the other major type of neurotransmitter receptor, the G protein-coupled receptor. G protein-coupled neurotransmitter receptors activate intracellular signaling pathways that produce a more slow and sustained response to neurotransmitter stimulation. In general, these receptor-mediated pathways serve to modulate neurotransmission by ion channels, maintain and mediate changes in protein expression, and promote cell survival. Most neurotransmitters have both ion channel receptors and G protein-coupled receptors, although a few, like dopamine and norepinephrine, bind only to G protein-coupled receptors.

Neurotransmitters stimulate receptors on postsynaptic membranes, but the signals receptors send are not always excitatory to the receiving neuron. Receptors, directly or indirectly, modulate excitability of the postsynaptic neuron, so that it is more or less likely to fire an action potential. For example, the neurotransmitter glutamate binds to both ion channel receptors and G protein-coupled receptors, and in each case these receptors transmit a signal that enhances the excitability of the receiving neuron. On the other hand, the neurotransmitter GABA (for *g*amma-*a*mino *b*utyric *a*cid), while also binding to both types of receptors, is known for its ability to decrease the excitability of the postsynaptic neuron. Its message is therefore inhibitory to the propagation of signaling within a group of neurons.

16.2.3 Glial Cells

While neurons constitute the definitive unit of the nervous system, their function is critically dependent on the presence of glial cells. In fact there are far more glial cells

in the nervous system than neurons. Glial cells perform many functions, including structural support, insulation, buffering, and guidance of migration during development. One class of glial cell, called microglia, is responsible for phagocytosis of cellular debris following injury and infection. The other types of glial cells, collectively known as macroglia, are the astrocytes and oligodendrocytes found in the CNS, and the Schwann cells found in the PNS. Oligodendrocytes and Schwann cells form myelin by wrapping multiple layers of plasma membrane around axons. Astrocytes are the most numerous of the glial cells, and they help form the blood-brain barrier, take up excess neurotransmitter and ions, and probably have some nutritive function. Metabolic enzymes expressed within astrocytes also regulate neuronal signaling by catabolizing excessive amounts of neurotransmitter. Monoamine oxidases, for example, catalyze the biotransformation of dopamine, norepinephrine, and serotonin into oxidation products that are substrates for further enzymatic reactions en route to excretion. The incidental bioactivation of the xenobiotic MPTP to its neurotoxic metabolite MPP+ by these enzymes will be discussed later in this chapter.

16.2.4 The Blood-Brain Barrier

The blood-brain barrier was conceptualized when it was noted that dyes injected into the bloodstream of animals stained nearly all tissues except the brain. It is thus this barrier and its PNS equivalent, the blood-nerve barrier, that prevents all but a select few molecules from entering the nervous system. The barrier itself is not a single unitary structure, but a combination of unique anatomical features that prevents the translocation of blood-borne agents from brain capillaries into the surrounding tissue. As mentioned above, astrocytes help form the barrier, surrounding capillary endothelial cells with extensions of their cytoplasm known as end-feet. There are also pericytes, whose function is not well known, that associate with the capillaries and may help induce a functional barrier. Another component of the barrier is the impermeable nature of the endothelial cells that line the interior of capillaries. Capillary endothelial cells in the nervous system are different from those in the periphery in at least three ways. First, brain capillaries form tight junctions of very high resistance between cells. In contrast, peripheral capillaries have low resistance tight junctions, and even openings, or fenestrations, that allow compounds to pass between cells. Second, compared to peripheral endothelial cells, brain endothelial cells are deficient in their ability to transport agents by endocytic mechanisms. Instead, only small lipophilic particles can be passed transcellularly. For larger molecules, carrier-mediated transport mechanisms are highly selective, and allow only one-way transport. Third, there is an enzymatic barrier that metabolizes nutrients and other compounds. Enzymes such as gamma-glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase are more prevalent in cerebral microvessels than in nonneuronal capillaries. Most of these enzymes are present at the lumenal side of the endothelium, Additionally the P-glycoprotein (P-gp) drug efflux transporter is presently thought to exist at the lumenal membrane surface, although some scientists argue that P-gp is actually associated with astrocytes. Finally the CNS endothelial cell displays a net negative charge at its lumenal side and at the basement membrane. This provides an additional selective mechanism by impeding passage of anionic molecules across the membrane.

Most of the toxicants that enter the nervous system do so by exploiting mechanisms designed to allow entry of essential molecules, such as nutrients, ions, and

neurotransmitter precursors. Small, lipophilic molecules are able to cross the blood-brain barrier relatively easily. Some agents can be recognized by active transport systems and thereby traverse the blood-brain barrier along with endogenous ligands. The Parkinson's disease therapeutic agent levodopa enters the brain in this manner. In some cases the blood-brain barrier is itself subject to damage by neurotoxicants. For example, lead, cadmium, mercury, and manganese accumulate in endothelial cells and damage their membranes, leading to brain hemorrhage and edema.

16.2.5 The Energy-Dependent Nervous System

Nervous tissue has a high demand for energy, yet nerve cells can only synthesize ATP through glucose metabolism in the presence of oxygen. Critical ATP-dependent processes in the nervous system include regulation of ion gradients, release and uptake of neurotransmitters, anterograde and retrograde axonal transport, active transport of nutrients across the blood-brain barrier, P-gp function, phosphorylation reactions, assembly of mitochondria, and many others. The highest demand for energy (up to 70%) is created by the maintenance of resting potential in the form of sodium and potassium concentration gradients across the nerve cell membrane. These gradients are maintained primarily by the activity of the Na^+/K^+ ATPase pump. The pump uses the energy of hydrolyzing each ATP molecule to transport three sodium ions out of the cell, and two potassium ions into the cell. Maintenance of the resting potential is not the only benefit of this pump's activity, however. The gradients created by the pump are also important for maintaining osmotic balance, and for the activity of indirect pumps that make use of the sodium gradient to transport other molecules against their own concentration gradient. Neurotransmission is thus heavily dependent on the proper functioning of the Na^+/K^+ ATPase pump.

Another process dependent on energy metabolism is axonal transport. Axonal transport carries organelles, vesicles, viruses, and neurotrophins between the nerve nucleus

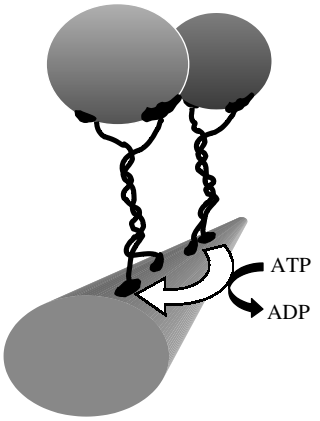

Figure 16.4 The microtubule motor protein kinesin. Kinesin consists of two ATP-hydrolyzing subunits that contact microtubules, a stem region, and regions that interact with vesicle and organelle proteins. One ATPase subunit binds and hydrolyses ATP, generating the force required to advance it forward. As this happens, the other subunit releases ADP, in preparation for binding another ATP, and its own advancement.

and the terminal. This distance can be quite long when one considers that the length of the sciatic nerve, for example, can be up to one meter. Anterograde transport (from cell body to terminal) is accomplished by two mechanisms defined by their rate, fast axonal transport and slow axonal transport. Fast axonal transport proceeds at rates of approximately 400 mm/day, and is mediated by the ATP-dependent motor protein kinesin. Kinesin forms cross-bridges between vesicles or organelles and microtubules, and dual projections of these cross-bridges shift back-to-front along microtubules in a coordinated, ATP-dependent manner, such that the entire molecule appears to be walking (Figure 16.4). Slow axonal transport is used to carry cytoskeletal elements such as tubulin and neurofilaments to the far ends of the axon, and it proceeds at approximately 0.2 to 5 mm/day. Traditionally slow transport has been regarded as passively dependent on axoplasmic flow; however, recent evidence suggests that the cytoskeletal elements actually move rather quickly but frequently stall in a stop-and-go fashion. Fast axonal transport also proceeds retrogradely, mediated by the ATP-dependent motor protein dynein. The rate of retrograde transport is about 200 mm/day. Neurons use retrograde transport for recycling membranes, vesicles, and their associated proteins. Neurotrophic factors, and some viruses and toxins (e.g., tetanus toxin) are also transported by this mechanism.

16.3 TOXICANT EFFECTS ON THE NERVOUS SYSTEM

Neurotoxicants affect the nervous system in a number of different ways. Some neurotoxicants damage the distal portions of axons without much effect on the remainder of the cell, while others produce outright cell death. Still others affect signaling processes in the nervous system, without causing structural damage. This wide variety of neurotoxicant effects is due in part to the unique nature of the different types of neurons and glia. Neurons may be differentially vulnerable to certain neurotoxicants because of their functional characteristics, as in the case of the targeting of substantia nigra neurons by the active metabolite of MPTP, an agent that causes Parkinson's disease. The substantia nigra, a brain region where neurons that synthesize dopamine are particularly abundant, sends out axons that project to other parts of the brain where the dopamine is released. After release, these neurons take back up the synaptic dopamine via selective transporters on the nerve endings. The damaging metabolite of MPTP, MPP+, is not distinguished from dopamine by the uptake transporter, so when present, MPP+ is taken up as well. MPP+ kills substantia nigra neurons by affecting mitochondrial energy production and promoting free radical formation. The death of these neurons results in a lack of dopamine release in an area of the brain called the stratum, which is responsible for the control of movement. The loss of dopamine in the stratum causes the hallmark symptoms of the neurodegenerative disease Parkinsonism: slowed movement, rigidity, and tremors. Because epidemiological studies have linked Parkinsonism in some patients with the agricultural use of pesticides, many of which are toxic to mitochondria, scientists believe that at least some cases of Parkinson's disease may be related to long-term exposure to environmental toxins.

MPTP is one example of a toxicant that causes direct structural damage to neurons, resulting in loss of function. In the following sections, other types of structural and functional effects of neurotoxicants are described. Structural effects are divided into three primary types: effects on myelin formation, primary damage to axons, and direct promotion of cell death. Neurons may also be secondarily affected by neurotoxicants

that target other cells in the nervous system, disrupting normal homeostatic function and causing structural or functional damage. Another method by which toxicants may affect the function of the nervous system is by directly altering synaptic neurotransmission.

16.3.1 Structural Effects of Toxicants on Neurons

Demyelination. The role of myelin in the nervous system is to aid in signal transduction. Myelin acts like an electrical insulator by preventing loss of ion current, and intact myelin is critical for the fast saltatory nerve conduction discussed above. Neurotoxicants that target the synthesis or integrity of PNS myelin may cause muscle weakness, poor coordination, and paralysis. In the brain, white matter tracts that connect neurons within and between hemispheres may be destroyed, in a syndrome known as toxic leukoencephalopathy. A multifocal distribution of brain lesions is reflected in mental deterioration, vision loss, speech disturbances, ataxia (inability to coordinate movements), and paralysis.

Demyelination occurs secondary to axonal degeneration, a topic covered in the section on axonopathy. Neurotoxicants that produce primary demyelination are uncommon, but may be divided into those that affect the integrity of the myelin sheath without or prior to damage to the myelinating cells, and those that directly injure myelin-producing cells. The former include agents like hexachlorophene, isoniazid, and the organotins. These compounds cause reversible edema between the layers of myelin by a mechanism that is yet unclear. The optic nerve is particularly susceptible to demyelination by hexachlorophene and organic solvents, whereas other cranial nerves, such as the trigeminal and vestibulocochlear, are vulnerable to styrene, xylene, and to trichloroethylene, an agent used in dry-cleaning. The metalloid tellurium damages myelin by inhibiting an enzyme involved in the synthesis of cholesterol, a major component of myelin.

In contrast to agents that target the integrity of the myelin sheath, chronic exposure to cyanide and carbon monoxide is thought to directly injure myelin-producing Schwann cell bodies in the PNS and oligodendrocytes in the CNS. Inorganic lead also causes direct damage myelinating cells. Oligodendrocytes appear more sensitive to lead toxicity than astrocytes or neurons. One mechanism for the devastating developmental effects of lead exposure may be the preferential inhibition of oligodendrocyte precursor cell differentiation.

Axonopathy. Axonopathy is a specialized form of neuronal damage, involving degeneration of the axon, while leaving the cell body intact. When axons die and degenerate, myelin breaks down as well, yet Schwann cells may survive and guide regeneration of the axon in some cases. Some toxicants produce axonal injury by directly targeting the axon itself. Others are thought to cause degenerative changes in axons by compromising the metabolic systems of the neuron. In the latter case the distal portion of the axon degenerates first, because it is this region that is most heavily dependent on intact axonal transport mechanisms. Since axonal transport is energy-dependent, toxicants that interfere with ATP production, such as the nicotinamide analogue Vacor, may cause distal regions to degenerate initially. Agents that target tubulin, like the vinca alkaloids, also cause this type of injury, because the tubulin-derived microtubules are critically important for axonal transport. With continued exposure the degeneration progresses

proximally, and eventually will affect the entire neuron. This distal-to-proximal degeneration is called "dying back neuropathy," and it affects the longest and largest diameter neurons most severely. If exposure to the toxicant is discontinued before death of the entire proximal axon and cell body, axons in the PNS will often regenerate, but axonal regeneration does not occur within the CNS. Regeneration in the PNS is dependent on Schwann cells proliferating and guiding growth of regenerating axon tips back to the target tissue.

In the 1850s Augustus Waller described the sequence of events that occurred following transection of a nerve fiber. These events have subsequently become known as Wallerian degeneration. The essential features of this type of degeneration include swelling of the axon at the distal end of the proximal segment of the transected axon, distal axonal dissolution and phagocytosis by inflammatory cells, and dissolution of myelin, with preservation and proliferation of Schwann cells along the length of the former axon. Certain neurotoxicants are capable of chemically transecting an axon, producing Wallerian degeneration similar to that occurring after slicing the nerve in half. Hexane, for example, forms covalent adducts with neurofilament proteins. This chemical crosslinking is thought to result in neurofilamentous axonal swellings that essentially block transport to regions of the axon distal to the swelling. The distal regions then die due to lack of communication with the neuron cell body, undergoing Wallerian degeneration.

Axonopathy can manifest as defects in sensory or motor functions, or a combination of the two. For most neurotoxicants, sensory changes are noticed first, followed by progressive involvement of motor neurons. One historically important case that illustrates these effects is that of the epidemic poisoning resulting from the consumption of "Ginger Jake" during Prohibition. Extracts of ginger used in tonics were legally required to contain 5 grams of ginger per milliliter of alcohol. The Department of Agriculture checked for compliance with this requirement by sampling the tonics, boiling off the ethanol, and weighing the solid content. Bootleggers soon discovered that a good deal of money could be saved by cutting back on the ginger and substituting it with adulterating agents like castor oil and molasses. It was such an attempt at adulterating Ginger Jake that led to the addition of Lyndol, a triorthocresyl phosphate (TOCP)-containing oil used in lacquers and varnishes, to tonic that was consumed by hundreds of thousands of people. The earliest signs that developed in people who had consumed the product were noted days to weeks later, and began with tingling and numbness in the hands and feet. In many, this progressed to leg cramps, weakness of the legs and arms, and ataxia. Those with minor symptoms improved, but perhaps thousands of people were left permanently paralyzed by the incident. Today TOCP is used to study the syndrome of delayed effects caused by some organophosphate compounds, commonly known as organophosphate-induced delayed neuropathy (OPIDN). The nature of OPIDN is still poorly understood. It appears not to be associated with organophosphate inhibition of acetylcholinesterase, but rather with another neuronal enzyme, the neuropathy target esterase (NTE). The physiological role of NTE is unknown.

Neuronopathy. Neuronopathy refers to generalized damage to nerve cells, with the primary damage occurring at the nerve cell body. Axonal and dendritic processes die secondarily in response to loss of the cell body. Like other cells in the body, neurons die by one of two processes distinguished by their morphological and molecular features: apoptosis and necrosis. (This division is overly simplistic; there is much debate

over the characteristics of the two categories, and whether there are more than two categories of cell death. Nevertheless, only these two will be considered here.) Often the same neurotoxicants can promote either form of cell death, depending on the intensity of the insult. For example, methylmercury given to rats at a high dose for one week causes widespread histopathological damage consistent with necrosis, whereas lower doses spread out over a longer time period results in apoptotic changes restricted primarily to cerebellar granule cells. It is thought that the severe and abrupt loss of cellular energy production by impairment of mitochondrial activity and plasma membrane disruption are responsible for necrotic death of neurons. This affects surrounding tissue more than apoptosis, since the dying cells release their contents and localized inflammatory responses ensue. On the other hand, apoptosis is death that is encoded within individual cells. Apoptosis is characterized by a process of cell shrinkage, pyknosis and fragmentation of nuclei, and membrane budding. The dying cell breaks apart into small membrane-bound apoptotic fragments that are phagocytosed, and thus collateral damage is reduced because only cells with activated death programs are affected. It is important to remember that apoptotic and necrotic mechanisms of cell death can occur concomitantly or sequentially, and thus are part of a continuum of effects associated with dose-dependent alterations in cellular energy production and the differential sensitivity of neuronal subtypes.

Many neurotoxicants produce their effects by promoting cell death in neurons. Neurotoxicant-induced cytotoxicity has been associated with the pathogenesis of a number of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), and the weight of evidence suggests that toxicant exposure is a risk factor for these diseases. One area of intense research focus has been the toxic effects of excessive signaling by glutamate and other excitatory amino acids (EAAs), and the role that EAAs may play in neurodegenerative disorders. Glutamate activates ion channel receptors that open to allow influx of calcium and other ions into the neuron. This influx of ions, combined with other second messenger events that promote further intracellular release of calcium, contribute to calcium overload. Signaling cascades are then activated in response to the intracellular calcium, and these pathways eventually lead to activation of oxidative stress and programmed cell death (apoptotic) pathways. EAA-mediated neuronopathic injury has been extensively studied for its role in ischemic and seizure-induced brain damage. It is furthermore thought that the glutamate receptor agonist, domoic acid, a toxin produced by algae, is responsible for several outbreaks of shellfish poisoning. In one incident in 1987, several people died, and dozens became ill with dizziness, seizures, and memory loss after consuming blue mussels. The mussels were contaminated with domoic acid, found in high levels following an algae bloom near Prince Edward Island, Canada. More recently domoic acid produced by algae blooms has been blamed for the abnormal behavior and deaths of pelicans, cormorants, and sea lions on the California coast.

16.3.2 Effects of Toxicants on Other Cells

Toxicants may selectively target glial cells for a number of reasons. Myelinating glial cells constantly synthesize cholesterol and cerebroside for myelin production; thus toxicants that affect these synthetic pathways will preferentially affect myelination. The hydrophobic nature of myelin may serve as a reservoir for lipophilic toxicants

as well. The specialized structures of cells that form myelin also present unique challenges to cellular homeostasis, increasing the vulnerability of these cells to toxicant action.

In general, a toxic or physical insult to either neurons or glial cells eventually leads to changes in the other cell type. Because of this, it is often difficult to determine whether the primary insult was neuronal or glial. Metals such as lead, cadmium, and aluminum are capable of inducing cell death in cultured astrocytes and endothelial cells. Methylmercury preferentially accumulates in astrocytes and to some extent in microglia, causing cellular swelling. The swelling is presumably the effect of methylmercury interfering with ion channels, because ion channel blockers can reverse this effect. Astrocytes are important reservoirs of excess glutamate, and swollen astrocytes release glutamate in and around synapses, potentially causing the excitotoxicity described above. Astrocyte swelling also has effects on brain blood flow, since astrocyte end-feet surround the blood vessels of the CNS. Not only does swelling result in reduced lumen size, but the distances substrates and waste products must diffuse to reach the bloodstream is increased.

Astrocytes and microglial cells become activated secondarily to brain injury, such as acute trauma or toxic lesioning. This activation, known as "reactive gliosis" is characterized by astrocyte hypertrophy and hyperplasia. Reactive astrocytes have greatly enlarged cytoplasmic processes, and produce increased amounts of a protein known as glial fibrillary acidic protein (GFAP). GFAP is often used as a quantitative histochemical marker for toxicant-mediated injury in the nervous system.

In addition to their function as phagocytic cells, glial cells produce neurotrophic factors to prevent neuronal death and promote axonal growth after injury. Glial cells also have xenobiotic biotransforming enzymes, but in some cases glial metabolism results in xenobiotic activation to a toxic metabolite. As discussed above, MPP+ is the toxic metabolite of MPTP. MPTP is taken up by astrocytes, where monoamine oxidase B converts MPTP to MPP+. While MPP+ seems to cause no damage to the astrocytes themselves, the astrocytes release this reactive metabolite into synapses, where it is selectively taken up by dopamine re-uptake transporters on the endings of neurons that normally release and recycle dopamine. MPP+ then kills the dopaminergic neurons, and this insult to the movement-controlling brain circuitry results in the classic motor symptoms of Parkinson's disease. The properties of MPTP have only become known since the 1980s, when its accidental ingestion by heroin addicts resulted in acute Parkinson-like symptoms. These incidents spurred multiple investigations, leading to much of what is known today about the pathogenesis of Parkinson's disease.

16.3.3 Toxicant-Mediated Alterations in Synaptic Function

Nervous system function may be adversely affected by neurotoxicants without necessarily causing structural damage to tissue. In many cases neurotoxicants interfere with signaling processes within the nervous system by activating or inhibiting receptors, or altering the amount of neurotransmitter available to activate receptors. This type of neurotoxicity is illustrated by the well-characterized actions of the organophosphates and carbamates on acetylcholine signaling.

Organophosphates inhibit acetylcholinesterase, the enzyme responsible for breaking down acetylcholine into acetic acid and choline. After acetylcholine has been

released into the synapse or the neuromuscular junction, acetylcholinesterase terminates receptor-stimulating activity by binding acetylcholine in its active site. Separate sites within the binding pocket of acetylcholinesterase bind the quaternary nitrogen of the choline group, and the carbonyl of the ester group. A hydrolytic reaction results in the loss of choline, leaving an acylated serine residue, which is then rapidly hydrolyzed. The biologically active oxon forms of organophosphates also bind to the active site of acetylcholinesterase, covalently phosphorylating the serine residue in the catalytic site of the enzyme. The phosphorylation of acetylcholinesterase creates a relatively stable inactive enzyme that persists for hours to days before hydrolysis of the phosphate moiety occurs spontaneously, and acetylcholinesterase activity is restored. The rate of spontaneous hydrolysis is increased with larger alkyl groups attached to the phosphate moiety. When one or more of these alkyl groups is lost, in a process known as "aging," spontaneous reactivation of acetylcholinesterase by hydrolysis of the phosphate moiety is impossible, and the enzyme is permanently inactivated. Carbamates similarly inhibit acetylcholinesterase by carbamylating the enzyme active site. The stability of carbamylation is much less than phosphorylation, however, and spontaneous reactivation thus occurs faster than with organophosphates.

The effects of acetylcholinesterase inhibition can be seen throughout the nervous system. Acetylcholine and its receptors mediate neurotransmission in sympathetic and parasympathetic autonomic ganglia, in the effector organs where autonomic nerves terminate, in neuromuscular junctions, and in the brain and spinal cord. The signs of hypercholinergic activity are thus very diverse, and include effects mediated by both nicotinic and muscarinic types of acetylcholine receptor. Hyperstimulation of nicotinic receptors in neuromuscular junctions results in muscle weakness, in rapid, localized contractions called fasciculations, and in paralysis. Nicotinic receptors are also found in sympathetic and parasympathetic ganglia, and so stimulation of both divisions of the autonomic system is apparent as hypertension, increased heart rate, and papillary dilation. Muscarinic receptors in the PNS mediate postganglionic parasympathetic effects on the smooth muscle present in the end organs such as the lung, gastrointestinal tract, eye, bladder, and secretory glands. Hyperstimulation of these receptors results in a pattern of toxicity known by the mnemonic SLUDGE (salivation, lacrimation, urination, defecation, GI upset, emesis). Bronchospasm and bradycardia are also muscarinic effects. In the CNS, confusion, anxiety, restlessness, ataxia, seizures, and coma are effects of both muscarinic and nicotinic receptor overstimulation. Death generally occurs from respiratory paralysis.

Treatment for toxicity by organophosphates and carbamates is directed at counteracting hyperstimulation and regenerating acetylcholinesterase enzymatic activity. Atropine is a muscarinic receptor antagonist (it blocks acetylcholine from binding to the muscarinic receptor), and is used to counteract the effects of cholinergic overactivity. Atropine has no effect at the nicotinic receptor, however, so the skeletal muscular and some of the sympathetic effects of cholinergic hyperstimulation will remain after administration of atropine. Inhibition of acetylcholinesterase activity by organophosphates can be reversed by administration of oxime compounds (e.g., pralidoxime and 2-PAM). These compounds contain a quaternary nitrogen that binds to the choline binding site of acetylcholinesterase, positioning the oxime portion of the molecule near the esteratic site. Oximes are themselves reversible inhibitors of acetylcholinesterase, but their mechanism of organophosphate reversal is by attack of the covalent phosphoserine bond, releasing the phosphate group. Oximes are not effective on dealkylated or

"aged" enzymes, so they must be administered soon after organophosphate intoxication in order to be effective. They are also ineffective against carbamate-mediated toxicity, and some researchers believe they actually worsen carbamate effects by stabilizing the carbamylation of the enzyme.

Many biological toxins produce hyperstimulation of receptors by directly binding and activating them (agonism), or reduce receptor stimulation by prohibiting the endogenous ligand from activating them (antagonism). A number of snake and spider venoms, mushroom and plant alkaloids, affect nervous system function by these mechanisms. As the binding of receptors by these agents is usually reversible, their effects are reversible as well (although some may still cause death by massively altering neuronal signaling). Beyond the receptor, an active area of current research is the role of intracellular signaling molecules in mediating the effects of neurotoxicants. The effects of a number of metals, in particular, may be related to their ability to act as cofactors for proteins involved in intracellular signaling. To date, however, few signal transduction molecules have been shown to be directly affected by neurotoxicants. Notable exceptions are cholera and pertussis toxins, which selectively target G proteins, but their primary effects are on the gastrointestinal and respiratory systems, respectively, rather than on the nervous system.

On the other hand, the Clostridium toxins, botulinum (causing botulism) and tetanospasmin (causing tetanus), block neurotransmission by inhibiting release of neurotransmitter into synapses and at motor end-plates in muscle. Both of these agents are structurally similar proteases, but the effects they cause are vastly different. Botulinum toxin enters presynaptic motor neurons in the PNS, where it cleaves proteins that are involved in the fusion of synaptic vesicles with membranes. This cleavage results in the inhibition of acetylcholine release from the presynaptic terminal, and thus muscles cannot be stimulated to contract. The clinical result of botulinum intoxication (usually by ingestion) is a flaccid paralysis. Recovery occurs when the presynaptic neuron sprouts new nerve endings that contact the muscle and create new motor end-plates. Tetanus toxin causes a completely different clinical picture, even though its substrate specificity for cleavage of proteins is very similar. Once taken up into the presynaptic nerve endings, tetanus associates with endosomes, and like endosomes, is transported retrogradely toward the neuron cell body. Tetanospasmin then continues its trek to the dendritic regions of the neurons, where it is released, again retrogradely, into synapses. Usually these synapses are within the spinal cord, where interneurons send an inhibitory signal (via the neurotransmitters glycine and GABA) to motor neurons to slow their activity and prevent massive muscular contraction. The presynaptic membranes of the interneurons take up tetanospasmin, and this is where most of its activity occurs. By cleaving release-regulating proteins in the interneuron terminal, tetanospasmin prevents the release of glycine and GABA onto the motor neurons. The motor neurons then become hyperactive, and this results in overstimulation of the motor end-plate with acetylcholine. Clinically this results in a spasms, stiffness, and whole-body paralysis. Again, the interneurons themselves do not die, but they must form new synapses with the motor neurons. Fortunately, in all but the most severe cases, recovery is complete. The reformation of new synapses by neurons, even in the CNS, is an example of the remarkable plasticity of the nervous system. The continual formation and reformation of synaptic connections allows the organism to change and adapt to an inconstant environment.

16.4 NEUROTOXICITY TESTING

A large number of the chemicals used in industry today remain poorly characterized with respect to their toxic effects on the nervous system. In order to determine potential risks to human and environmental well-being, existing neurotoxicants must be identified, and the approximately 2000 new chemicals introduced each year must be screened for their potential neurotoxic effects. Often, a tiered approach is used, with the first tier consisting of general screening tests to identify acute hazards. The Environmental Protection Agency (EPA) has proposed screening guidelines for tests in rodents that include a functional observational battery (FOB, see below) to evaluate sensory, motor, and autonomic effects, tests that identify changes in motor activity, and neuropathological assessment. Interpretation of the outcome of tier 1 screening may lead to more selective testing and examining the effects of repeated exposures in the second tier. Specialized tests for behavioral effects, developmental neurotoxicity, or delayed organophosphate effects may be required. If necessary, a third tier of testing characterizes mechanisms of neurotoxicant-induced injury. Complete and comprehensive evaluation of potential neurotoxicant effects requires that data from different types of sources be considered; this can range from molecular interactions to whole animal and human exposure analysis. Below are examples of techniques commonly used for testing neurotoxic effects.

16.4.1 In vivo Tests of Human Exposure

Historically the first indication of neurotoxic potential by a chemical has often followed accidental human exposure in the workplace. Case reports of incidents involving individuals, or clusters of individuals, are useful for documentation but generally provide a limited amount of information about the specific details of an exposure. Procedures included in most case reports include a patient medical history and clinical neurological exam, sometimes supplemented with psychiatric or neurophysiological tests, and/or neuroimaging. Although the specific tests involved vary depending on the clinician, most basic clinical neurological exams rely heavily on evaluation of mental status (level of consciousness, orientation, mood, etc.) and sensorimotor function (gait, coordination, muscle tone, sensitivity to touch, reflexes).

Human epidemiological studies generally represent a deeper investigation into the causal relationship between an exposure and neurotoxicological effects. Some of the methods used to identify neurotoxic effects in epidemiological studies include behavioral assessments, neurophysiological evaluations, and neuroimaging techniques. Neurobehavioral assessments examine a variety of psychological and cognitive functions such as mood, attention, memory, perceptual and visuospatial ability, and psychomotor performance. In an effort to standardize neurotoxicological testing of human behavioral effects, particularly for studies involving worksite exposure, the World Health Organization (WHO) and the US National Institute for Occupational Safety and Health (NIOSH) devised a the Neurobehavioral Core Test Battery (NCBT). The NCBT (Table 16.1) consists of seven tests that were shown previously to be sensitive indicators of neurotoxicant exposure. The battery is designed to be administered one on one by an examiner. Although this battery has a relatively narrow

Table 16.1 The WHO Neurobehavioral Core Test Battery (NBCT)

Domain	Analysis	Test	Task
Psychomotor performance	Motor speed, motor steadiness	Pursuit aiming	Follow a pattern of small circles, placing a dot in each circle around a pattern; subject's score is number of taps in circle within one minute.
	Manual dexterity, hand–eye coordination	Santa Ana Dexterity Test	Perform skillful movements with hands and arms.
Perceptual coding and perceptual motor speed		Wechsler Digit Symbol Test	Each number in a list is associated with a simple symbol. On a list of random digits with blank spaces below them, write the correct symbols in blank spaces as fast as possible.
Attention and short-term memory	Attention and response speed	Simple reaction time	Test reactions of hands or feet from visual and auditory signals.
	Visual perception and memory	Benton Visual Retention Test	Recall and reproduce figures.
	Auditory memory	Wechsler Digit Span Test	Recall digits in series forwards and backwards immediately after hearing them.
Mood and affect		Profile of Mood States	Evaluate, by questionnaire, anger, tension, confusion, depression, etc.

focus, primarily on the effects most commonly seen in CNS toxicity, it also provides suggestions for the selection of further testing depending on the exposure setting. The NCBT has been widely used because of its ease of administration, relatively low cost, and its large base of control data. A broader battery of cognitive and psychomotor tests that is often used is the Neurobehavioral Evaluation System (NES). The NES consists of a combination of automated (computerized) and hand-administered tests. The sensitivity of the NES to effects caused by neurotoxicants in industrial settings has been validated internationally.

Neurobehavioral examinations are useful for identifying neurotoxicant-mediated deficits, but it is often difficult to localize the site of toxic action from such tests. For example, sensorimotor tests of reaction time, manual dexterity, hand-eye coordination, and finger tapping can indicate either neuromuscular or psychomotor damage.

The results of these tests thus should be interpreted in the context of other experiments. For example, electrophysiological techniques can help to focus an investigation to the site of the lesion, and characterize electrical dysfunction within the damaged nerves. Electrophysiological nerve conduction studies can distinguish between proximal and distal axonal lesions in peripheral nerves and can be performed noninvasively (i.e., with skin surface electrodes). Characteristic changes in the velocity, duration, amplitude, waveform, or refractory period of peripheral nerves may be detected, depending on the agent. Evoked potentials represent another useful electrophysiological endpoint. These procedures measure the function of an entire system, such as the visual, auditory, or motor systems. The specific pathway is stimulated by an evoking stimulus, such as a flash of light or electrical nerve stimulation. The evoked potentials are read as changes in ongoing electroencephalograms (EEGs) in response to the stimulation. Thus the activity of the entire neural circuit is evaluated in the brain after peripheral stimulation. Evoked potentials can be very sensitive indicators of changes in neural activity when performed in a carefully controlled environment, and when interpreted in light of behavioral or other physiological findings.

An increasingly popular method of documenting brain pathology is the use of neuroimaging methods. Computerized axial tomography (CAT) and magnetic resonance imaging (MRI) can produce images of the brain that can show structural changes in the volume or density of a specific region or ventricle. Other techniques, such as positron emission tomography (PET) and single photon emission computerized tomography (SPECT), use radioactive tracer molecules to determine functional biochemical changes in processes like glucose utilization or receptor binding. The number of cases so far analyzed with neuroimaging techniques is still relatively small, and thus specific toxicant-mediated effects are not well characterized. Nevertheless, this growing field promises to contribute significantly to neurotoxicity studies in the future.

16.4.2 In vivo Tests of Animal Exposure

The primary approach currently used to detect and characterize potential neurotoxicants involves the use of animal models, particularly rodents. Behavioral and neurophysiological tests, often similar to the ones used in humans, are typically administered. The sensitivity of these measures to neurotoxicant exposure is widely accepted. Although it is often not possible to test toxicant effects on some higher behavioral functions in animals (e.g., verbal ability, cognitive flexibility), there are other neurobehavioral outcomes such as memory loss, motivational defects, somatosensory deficits, and motor dysfunction that can be successfully modeled in rodents. These behaviors are based on the ability of the nervous system to integrate multiple inputs and outputs, thus they cannot be modeled adequately in vitro. Although the bulk of neurotoxicity data has been collected in rodents, birds and primates are also used to model human behavioral outcomes.

As mentioned above, a useful screening tool for neurotoxicant exposure is a battery of observational tests of function known as an FOB. FOBs, like the one developed by the EPA, are used to detect overt changes in behavior and physiology of animals exposed to neurotoxicants. In the typical exam, an observer documents cageside observations regarding the appearance and activity of the animal. Then the animal is handled and examined for obvious signs such as lacrimation, salivation, or piloerection. Pupillary light responses and temperature are recorded, and the ease of handling the animal

is also noted. The animal is then placed in an open field, such as the top of a laboratory cart, and observed for a set period of time, during which the observer records exploratory behaviors, excretion rate, and whether or not there are any motor abnormalities. A number of manipulations are then performed to assess hearing, sensitivity to touch, righting reflex, coordination, and grip strength. The FOB used in the EPA guidelines has shown remarkable consistency in detecting chemical effects in diverse testing laboratories and situations. A test of motor activity can be administered along with the FOB, consisting of quantitative evaluations of the animal's movement in either an open field or a maze. Motor activity tests reflect integrative abilities of the nervous system to process sensory input, association, and motor output. A number of agents, such as toluene, triadimefon, and chlorinated hydrocarbons, increase or decrease motor activity in a toxicant-specific manner, unrelated to their general effects on the health of the animal.

More in-depth behavioral tests are required if dose-related toxicant effects are noted in screening tests. These tests may also be required as part of more selective toxicological screening, such as for developmental neurotoxicity. Focused tests of neuromotor function and activity, sensory functions, memory, attention, and motivation help to identify sites of toxicant-mediated lesioning, aid in the classification of neurotoxicants, and may suggest mechanisms of action. Some of these tests, like the schedule-controlled operant behavior tests for cognitive function, require animal training and extensive operator interaction with the animals.

16.4.3 In vitro Neurochemical and Histopathological End Points

In vitro methods for studying neurotoxicant effects are a valuable supplement to whole animal and human testing, allowing the researcher to supplement findings, test hypotheses, and reduce the number of animals used for toxicity testing. Much of the neurochemical and histopathological data on neurotoxicant effects in humans and animals is gathered concomitantly with, or immediately after, performing behavioral tests. This may involve collection of bodily fluids or samples from living subjects for the purpose of analyses such as red blood cell or plasma acetylcholinesterase or NTE activity, determination of hormone or neurotransmitter concentration, or detection of the presence of toxicant or metabolite in the cerebrospinal fluid.

Postmortem tissues can provide a wealth of information about the location, timing, extent, and mechanism of neurotoxicant-induced damage. For example, changes in the gross morphology and weight of brain or nerves may be seen at higher levels of toxicant exposure. Microscopically, fixed and stained tissues reveal the type of damage to target cells, such as axonopathic or demyelinating lesions. Degenerative changes in cells may be indicative of the injurious process, and whether cells are dying by necrosis or apoptosis. Typical stains such as cresyl violet (Nissl stain) and silver (Golgi stain) are useful for cell morphology and counting. Other stains are selective for damaged cells, like the specialized silver impregnation techniques that are frequently used to identify neurotoxicant-mediated degeneration of neurons.

Tissue sections may also be processed for immunohistochemical staining. A frequently used immunochemical marker for neuropathologic insult is glial fibrillary acidic protein (GFAP). GFAP is produced in large amounts by reactive astrocytes that proliferate in response to tissue injury. Stress proteins, apoptotic signals, and immediate early genes are also utilized as markers of neuronal activity and injury. Other protein markers

can be used to quantitatively identify specific types of neurons, which may be reduced in numbers after selective neurotoxicant-induced cell death. For example, tyrosine hydroxylase (TH) is an enzyme involved in dopamine synthesis and, as such, is selectively expressed in dopamine-containing neurons. Loss of TH immunoreactivity is used to identify dopaminergic cell death after administration of the neurotoxicant MPTP.

In homogenized tissue preparations, mechanistic information can be obtained from analyzing tissue levels of neurotransmitter and metabolites, signaling proteins, and receptor-binding affinities. Protein and lipid peroxidation and oxygen radical formation are commonly seen with toxicants that target mitochondrial function. Neurotoxicants may alter the levels or activation state of many proteins, including kinases, phosphatases, and proteases, quantifiable with activity or immunological techniques.

Cell culture protocols are a useful adjunct to neurotoxicity testing. Individual cell lines are particularly well suited for identifying selective cellular and molecular toxicity and for studying the mechanistic aspects of neurotoxicant injury. Clonal cells lines, as well as primary cultures of neurons or glial cells, may be used, and the choice of cell type or particular clonal line depends on the particular end points under study. For example, if a researcher wished to study the effects of a given neurotoxicant on neurotransmitter release, she might choose the rat pheochromocytoma PC12 cell, which releases catecholamine neurotransmitter upon stimulation with a variety of agents. The relative inexpensive and ease of manipulating exposure make cellular techniques an attractive alternative for many types of studies. Cultured cell studies cannot, however, reproduce systemic metabolic and kinetic effects, or mimic the complex neuronal circuitry that is present in vivo. Thus, while cell studies provide a vehicle for in-depth examination of the nature of toxicant-cellular interactions, extrapolation to in vivo conditions is often not possible.

16.5 SUMMARY

The nervous system is at once unique in structure and staggeringly complex, exquisitely sensitive, yet capable of amazing adaptability. Because of these attributes, the neurotoxic potential of many agents, to say little of their underlying mechanisms, remains unknown. Particularly concerning are the possibilities that chronic low levels of chemical exposure are having an effect on the behavioral development of children, and contributing subtly to neurodegenerative diseases in the elderly. The huge task of testing natural and synthetic chemicals for neurotoxic effects has been facilitated in recent years with the development of behavioral testing batteries, advances in pathological and biochemical techniques, and a more focused attention of regulatory agencies on issues relating to neurotoxicology.

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CHAPTER 17

Endocrine System

GERALD A. LEBLANC

17.1 INTRODUCTION

Among the various organ systems of the body, the endocrine system is somewhat unique. While most systems are associated with a specific physiological task (respiration, reproduction, excretion, etc.), the endocrine system functions to regulate many of the activities associated with these other systems. Accordingly the endocrine system is integral to the maintenance of total normal bodily function (homeostasis), and disruption of normal endocrine function by exogenous chemicals can result in multiple, diverse, and dire consequences. Toxicity to the endocrine system is most commonly associated with altered development, growth, maturation, and reproduction (Table 17.1). However, endocrine toxicity also can present as gastro-intestinal dysfunction, malaise, neurological and other disorders (Table 17.1). This is why endocrine toxicity often can be misconstrued as toxicity to some other endocrine-regulated system of the body.

The endocrine system, as an authentic target of chemical toxicity, tragically entered the limelight as a consequence of the widespread use of the drug diethylstilbestrol (DES). DES, a nonsteroidal synthetic estrogen, was prescribed to pregnant woman from the 1940s to the 1960s as a prophylactic against miscarriage (see Section 17.4.1). Following the discovery of the endocrine toxicity of this drug, many additional drugs and environmental chemicals have been shown to mimic the action of hormones or interfere with their hormonal function. These activities often have been clearly shown, in laboratory studies, to result in endocrine-related toxicity. In some instances drug use or exposure to ambient environmental chemicals has been shown to result in endocrine toxicity. Such examples will be presented at the end of this chapter.

17.2 ENDOCRINE SYSTEM

The endocrine system can be broadly described as *an assemblage of organs (glands) that produce chemical messengers (hormones) that regulate various bodily functions*. The bodily functions regulated by the endocrine system can be categorized as those

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Table 17.1 Processes Regulated by Some Hormones of the Endocrine System Susceptible to Disruption by Endocrine Toxicants

Hormone Group	Example	Origin	Regulated Process
Androgens	Testosterone	Testes, adrenals	Sexual differentiation, fertility, secondary sex characteristics, sexual function, libido
Estrogens	17β-Estradiol	Ovaries, testes	Sexual differentiation, fertility, secondary sex characteristics, bone density maintenance, blood coagulation
Glucocorticoids	Cortisol	Adrenals	Bone formation, wound healing, growth, development
Thyroid hormones	Thyroxine	Thyroid gland	Fetal brain and bone development, oxygen consumption, gut motility

involved in the maintenance of homeostasis and those involved in physiological progression. Functions regulated by the endocrine system resulting in homeostasis include maintenance of the reproductive system, energy production, and metabolism. Functions regulated by the endocrine system resulting in physiological progression include fetal development, growth, and maturation. Endocrine processes related to physiological progression historically have received the greatest attention in endocrine toxicology and will be emphasized in this chapter.

Both the maintenance of homeostasis and the regulation of physiological progression require that the endocrine system detect signals, either external or internal, and transduce these signals to the appropriate target sites within the body. These target sites then respond in the appropriate manner to maintain homeostasis or institute change related to development, maturation, and so on. In many species these initial signals are of external origin. For example, many species initiate reproductive maturation in response to changes in environmental temperature and day length. Reproductively mature organisms often respond to external visual or olfactory stimuli produced by sexually receptive individual to initiate sexual behavior.

The signal to be transduced by the endocrine system initiates in the central nervous system. In mammals, the hypothalamus commonly initiates the endocrine signaling pathway by secreting peptide hormones. These neuro-endocrine hormones can be rapidly synthesized, secreted, and degraded to allow near-instantaneous, short-lived responses to the stimulatory signal. Accordingly they can be present in the body in pulses and secretory rhythms that often contribute to their signaling function. For example, the hypothalamic peptide hormones "growth hormone releasing hormone" (GHRH) and somatostatin are secreted in an alternating pulsatile fashion. Both hormones target the pituitary gland, though GHRH stimulates and somatostatin inhibits growth hormone secretion by the pituitary. As a result the secretory pattern of the secondary hormone messenger in this cascade, growth hormone, is highly controlled. Disruption of this rhythm in rodent models can alter hepatic enzyme expression and other dynamic processes. Disruption of the growth hormone secretory rhythm associated with sleep has been shown to interfere with normal growth in children. Hormone

secretory rhythms have been associated with other physiological processes including sleep, sexual behavior, and ovulation.

Endocrine signaling pathways from the central nervous system to the target organ typically occur along axes (Figure 17.1). An axis is defined by the endocrine glands that produce signaling hormones along the cascade (i.e., hypothalamic–pituitary–gonadal axis), and sometimes, a terminal target organ of the signaling pathway (i.e., hypothalamic–pituitary–gonadal–hepatic axis).

Endocrine signaling cascades offer several advantages over a single hormone signaling strategy. Cascades provide several sites at which the signal can be regulated thus ensuring maintenance of the appropriate endocrine signal (Figure 17.2). For example, testosterone is secreted by the testis but regulates its own secretion by acting upstream in the axis at the pituitary gland and hypothalamic gland. Signaling cascades also

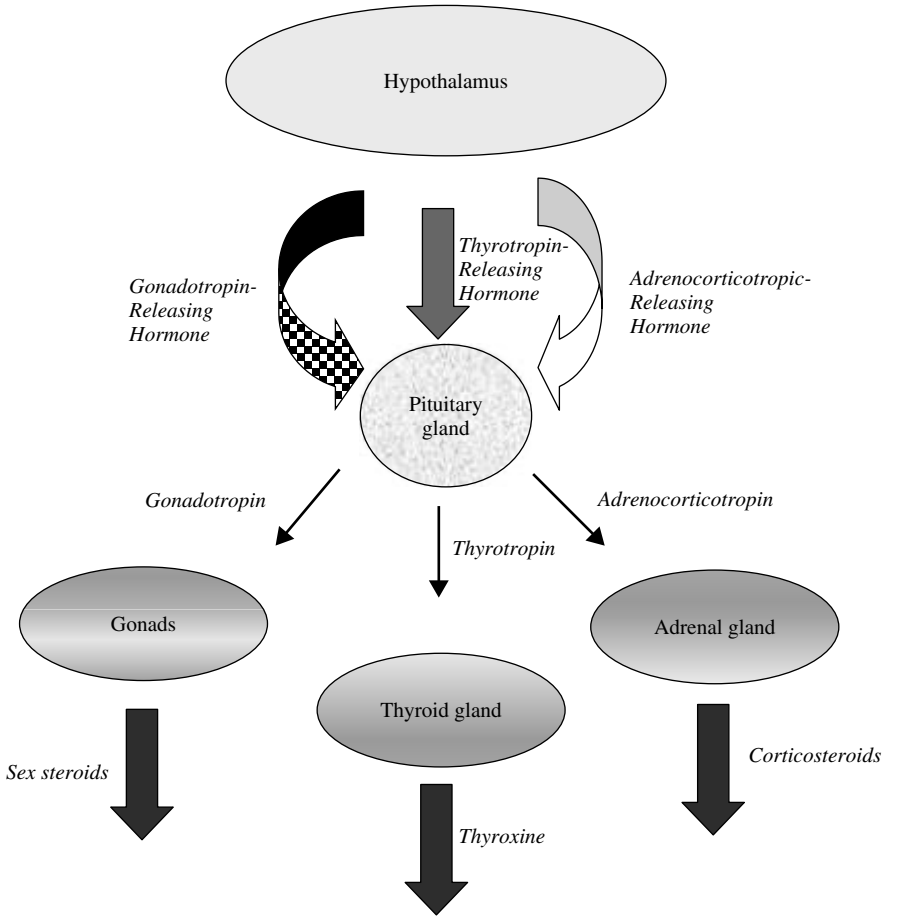

Figure 17.1 Some major neuro–endocrine axes that transduce endocrine signals to target organs. Neuro–endocrine signaling is initiated by the secretion of releasing hormones, or in some instances inhibiting hormones, that regulate secretion of the secondary hormone signal by the pituitary. Pituitary hormones then regulate secretion of the tertiary hormone, often a steroid hormone, by the appropriate endocrine gland. The tertiary hormones then stimulate gene transcription at target organs.

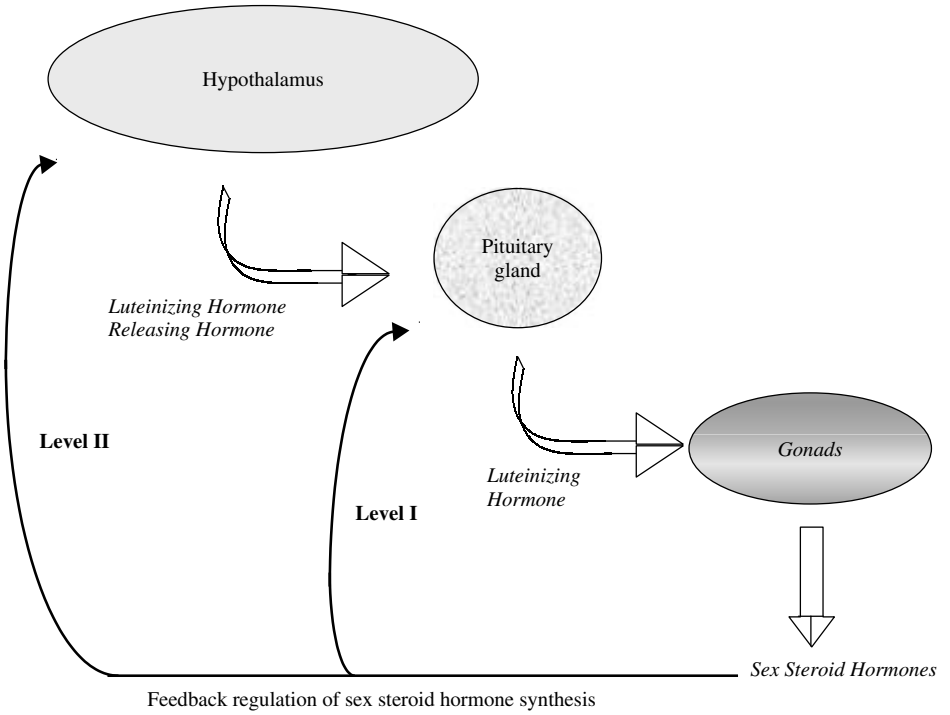

Figure 17.2 The hypothalamic–pituitary–gonadal axis. Endocrine signaling cascades provide multiple sites for regulation and ensure optimum signaling.

utilize multiple hormones with differing properties to contribute to the process. Peptide hormones are commonly the intermediate messengers along a signaling cascade, while the terminal hormone is often of nonpeptide origin (i.e., steroids). Peptide hormones offer advantages as intermediate messengers in that they can be rapidly synthesized and degraded (i.e., turned "on" and "off"). Peptide hormones also do not require cell entry to elicit activity but rather bind to cell surface receptors. This facilitates a rapid physiological response to the hormone. Steroid and other nonpeptide hormones are typically more stable, they are maintained in circulation at a relatively constant, physiologically appropriate level, they can be stored as precursor molecules or apolar conjugates, they can be mobilized as polar conjugates, and most often, they require cell entry to interact with its receptor and elicit a response. Accordingly the nonpeptide terminal hormones offer the advantages of constant availability but lack the advantages of rapid modulation.

17.2.1 Nuclear Receptors

Toxicologically the function of the terminal hormones of endocrine cascades (i.e., steroid, retinoid, thyroid hormones) appear to be most susceptible to disruption by chemicals. This is because many foreign molecules share sufficient characteristics with these hormone molecules to allow binding to the nuclear receptors of these hormones in either an agonistic or antagonistic fashion. The binding of the xenobiotic to the

receptor results in aberrant receptor function with associated toxicological outcome. The nuclear receptors are so called since these receptors initiate their classical physiological responses within the cell nucleus. Cell surface receptors to peptide hormones, on the other hand, can likely discriminate between peptide molecules and nonpeptide xenobiotics thus minimizing the likelihood of interaction and associated disruption of function.

The nuclear receptor superfamily consists of members of the steroid receptor family and the thyroid receptor family (Figure 17.3). Members of these two receptor families are distinct in many structural and functional attributes. Steroid receptor family members typically exist in the extranuclear matrix of the cell in association with various accessory proteins (*hsp*90, *hsp*70, *hsp*56). These accessory proteins stabilize the receptor molecule and help maintain the molecule's integrity. Binding of hormone ligand to the receptor protein stimulates dissociation with the accessory proteins, homodimerization of two receptor molecules, and nuclear localization (Figure 17.4). Here the receptor complex interacts with hormone response elements (Table 17.2) that are associated with hormone responsive genes, and transcription of these genes is regulated.

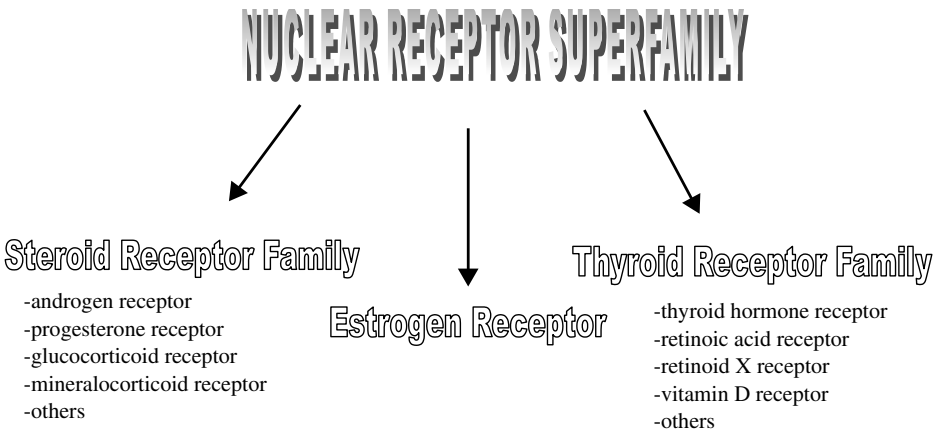

Figure 17.3 The nuclear receptor superfamily. Steroid receptor family members and thyroid receptor family members differ in several structural and functional properties. The estrogen receptors share properties with both steroid and thyroid receptor families and are likely an evolutionary precursor to both families.

Table 17.2 DNA Recognition Sequences Utilized by Steroid, Estrogen, and Thyroid Receptor Family Members

Receptor Family	Recognition Sequence
Steroid	AGAACA . . . TGTTCT
Estrogen	AGGTCA . . . TGACCT
Thyroid	AGGTCA . . . AGGTCA

Note: Recognition sequences are split by spacer nucleotides, which are denoted by the dots.

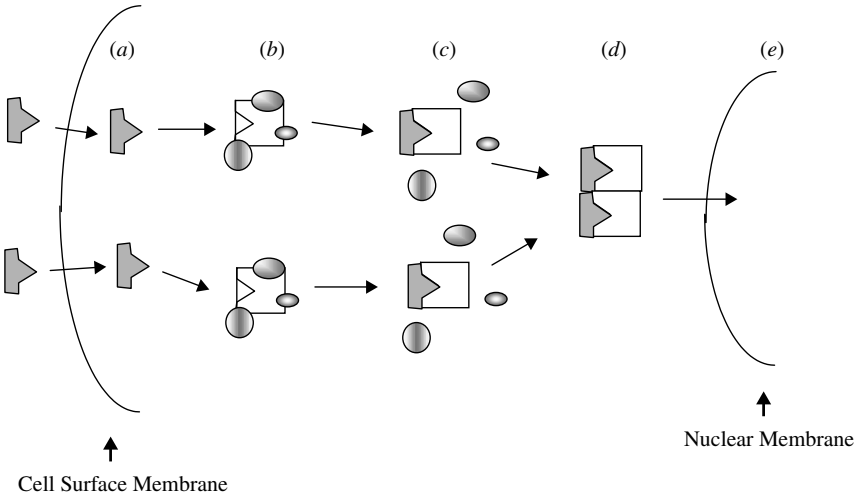

Figure 17.4 Intracellular steroid receptor activation by hormone ligands. (*a*) Steroid hormones diffuse across the cell membrane into the cell. (*b*) Steroid hormone receptors in the basal state bound to accessory proteins. (*c*) Steroid hormones bind to receptors and accessory proteins are dissociated from the receptors. (*d*) Hormone : receptor complexes dimerize. (*e*) Dimer complexes enter the nucleus and initiate transcription of responsive genes.

In contrast to the steroid receptor family, members of the thyroid receptor family typically do not associate with accessory proteins and are not localized to the extranucleus matrix. Rather, these receptors exist in the basal state associated with chromatin in the cell nucleus. When bound by hormone ligand, thyroid receptor family members dissociate from the chromatin and typically form heterodimeric combinations with the retinoid-X receptor (RXR). RXR also is capable of homodimerization in association with its ligand 9-*cis* retinoic acid. Thus high 9-*cis* retinoic acid levels apparently promote homodimerization, and low levels are permissive of heterodimerization of RXR with activation by the partner ligand.

The estrogen receptor shares structural and functional attributes of both nuclear receptor families. For example, the estrogen receptor resides in the cell nucleus, but it associates with accessory proteins rather than chromatin. The estrogen receptor shares a high degree of sequence homology with the thyroid receptor family members but does not heterodimerize with RXR. Rather, active estrogen receptor exists as a homodimer. The observation that the estrogen receptor shares attributes of both nuclear receptor families supports nucleotide sequence evidence that the estrogen receptor is an ancestral precursor to both receptor families.

17.2.2 Membrane-Bound Steroid Hormone Receptors

Some cellular responses occur too rapidly following steroid hormone exposure to involve the multi-step process of nuclear receptor activation. For example, 17β-estradiol can rapidly stimulate adenylate cyclase and cause a near-instantaneous increase in intracellular cAMP in cultured prostate cells. These effects are mediated by the interaction of steroid hormones with cell surface proteins.

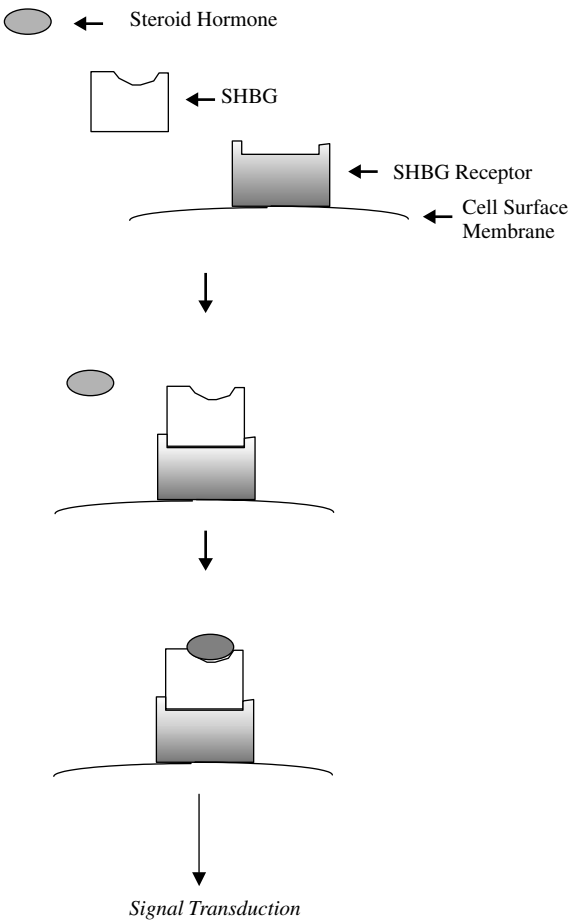

Figure 17.5 Endocrine signaling pathway involving steroid hormone, sex hormone binding globulin (SHBG), and the SHBG receptor.

Due to their lipophilic nature, steroid hormones are mobilized in the circulatory system by transfer proteins. Sex hormone-binding globulin (SHBG) is one such transfer protein that binds testosterone, 17β-estradiol, and other sex steroids. Roughly half of circulating testosterone and 17β-estradiol is bound to SHBG. Receptors exist on the surface of some cells that are capable of binding unliganded SHBG (Figure 17.5). Unliganded SHBG, which is bound to the cell surface receptor, can subsequently bind steroid hormone. Binding of an appropriate hormone to the SHBG then stimulates a signal-transduction pathway within the cell. Some steroid hormones (17β-estradiol, 5α-androstan-3α,17β-diol) function as SHBG : SHBG-receptor agonists, while others (testosterone, 5α-dihydrotestosterone) function as antagonists. Interestingly 5α-androstan-3α,17β-diol had previously been considered an inactivation product of the potent androgen 5α-dihydrotestosterone (DHT). Studies in human prostate cells have shown that activation of this SHBG-dependent pathway stimulates DNA synthesis and cell growth. These observations, in combination with studies in dogs that have shown 5α-androstan-3α,17β-diol to stimulate benign prostatic hyperplasia, have led to

suggestions that the SHBG-receptor pathway is involved in this disease condition. The susceptibility of these membrane-bound receptor pathways in endocrine toxicity has received little attention, though conceivably toxicants could perturb these pathways by competing with endogenous hormone for binding to SHBG, resulting in the loss of stimulatory activity (antagonists) or inappropriate stimulation of activity (agonists).

17.3 ENDOCRINE DISRUPTION

Xenobiotics have the ability to disrupt hormone activity through a variety of mechanisms, though the predominant mechanisms appear to involve binding to the hormone receptor, either as an agonist or antagonist, or by modulating endogenous steroid hormone levels.

17.3.1 Hormone Receptor Agonists

A hormone receptor agonist is defined as *a compound that binds to and activates a hormone receptor*. Endogenous hormones function as agonists to their respective receptors. Xenobiotics can act as receptor agonists and stimulate receptor-dependent physiological processes in the absence of the endogenous receptor ligand (hormone). Such inappropriate stimulation can result in the errant expression of hormone-dependent processes such as breast development in males (gynecomastia).

Estrogen Receptor. Among the steroid hormone receptors, the estrogen receptor appears most susceptible to the agonistic action of xenobiotics. Estrogen receptor agonists are quite diverse in molecular structure (Figure 17.6). Several steric considerations, associated with the steroid structure, in conjunction with electrostatic (charge) properties of the outer surface of the molecule seem to dictate whether a xenobiotic can fit into the binding-pocket of the receptor and function as a receptor agonist. It is not clear why the estrogen receptor would be more susceptible to the agonistic action of xenobiotics as compared to other steroid hormone receptors. High ligand specificity may be an evolutionary trait of most "modern" receptors that is not associated with this presumed ancestral precursor to other nuclear receptor superfamily members. The estrogen receptor is often referred to as a promiscuous receptor because of this susceptibility to agonistic interactions with xenobiotics.

Some drugs are rather potent estrogens (i.e., DES); however, environmental chemicals with estrogenic activity are typically weak agonists with activity several orders-of-magnitude less than that of 17β-estradiol (Table 17.3). Because of this weak activity, xenoestrogens are typically not associated with endocrine toxicity to adult females owing to the large amount of 17β-estradiol in these individuals. However, adult males, immature individuals, and embryos all have been shown to exhibit endocrine toxicity resulting from xeno-estrogen exposure. For example, in utero exposure of male or female rodents and humans to DES causes proliferation of epithelial cells associated with the reproductive system resulting in abnormalities of this system. Gynecomastia is a common side effect of estrogenic drugs such as DES and fosfestrol when administered to adult males. The physiological consequences of xeno-estrogenic activity is typically characteristic of feminization, that is, the acquisition of female characteristics.

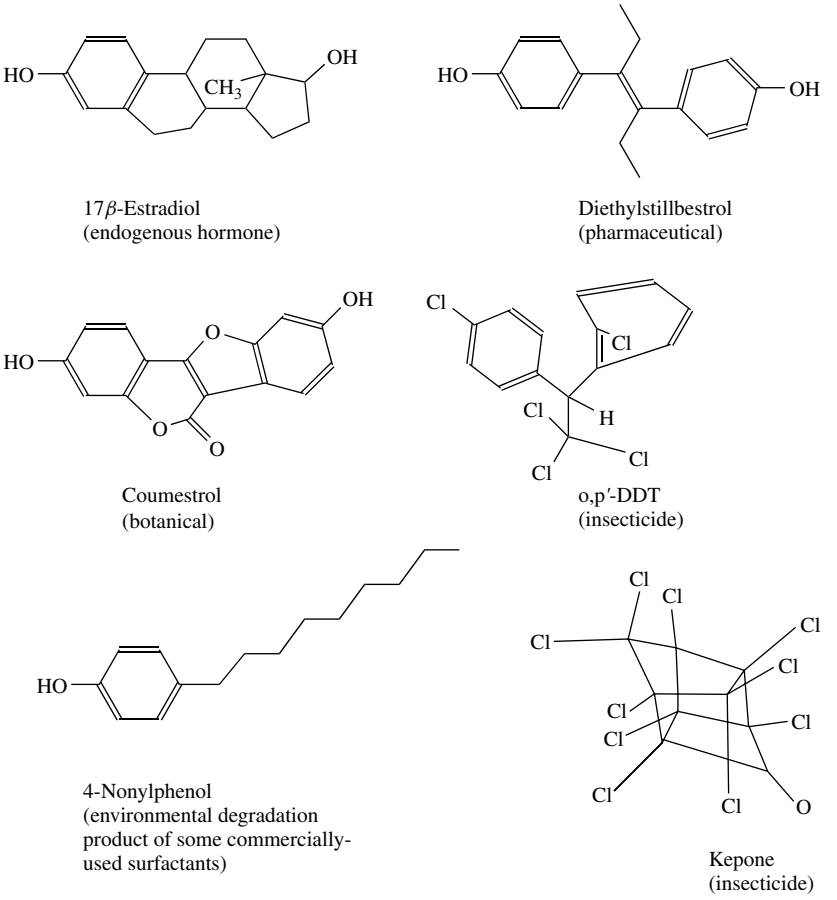

Figure 17.6 Estrogen receptor agonists. Molecules are presented in a low-energy state that may represent their natural three-dimensional conformation.

Ecdysone Receptor. Ecdysteroids are a class of steroid hormones that regulate a variety of processes related to development, growth, and reproduction in insects and other arthropods but are not utilized by vertebrates. Many compounds of plant origin, or derivations thereof, have been identified that are ecdysteroid receptor agonists (i.e., cucurbitacins, withasteroids). The ecdysteroid agonists are presumed to have evolved in plants as a means of protection against insect predation. Some environmental chemicals of anthropogenic origin also have been shown to exhibit ecdysteroid receptor agonistic activity (i.e., tebufenozide) and have been exploited as insecticides due to their ability to interfere with insect development and growth.

Retinoic Acid Receptor. Most of the biological effects of retinoids are mediated through the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Both all-*trans*-retinoic acid and 9-*cis*-retinoic acid serve as agonists of RAR, while only 9-*cis*-retinoic acid functions as an agonist of RXR. The functional RAR exists as a heterodimer with RXR, while functional RXR exists as a homodimer. Methoprene is a juvenile hormone III analogue that mimics the activity of this insect hormone.

Table 17.3 Potency of Some Xenoestrogens Relative to 17β-Estradiol

Chemical	Potency
17β-Estradiol	100
Diethylstilbestrol	74
4-Nonylphenol	0.005
4-Octylphenol	0.003
4-*tert*-Octylphenol	0.00036
o', p'-DDT	0.00011
o', p'-DDE	0.00004
2′, 5′-Dichloro-4-biphenylol	0.62
2′, 4′, 6-Trichloro-4-biphenylol	1.0
2′, 3′, 4′, 5′-Tetrachloro-4-biphenylol	0.82
Bisphenol A	0.005
Butylbenzylphthalate	0.0004

Source: Coldham, N. G. et al., 1997, *Environ. Health Perspect.* **105**(7): 734–742.
Note: Estrogenic potency of the compounds was measured using a recombinant yeast cell bioassay.

Exposure of juvenile insects to methoprene results in various abnormalities associated with development and ultimately death. The environmental degradation product of methoprene, methoprenic acid was found to serve as an RXR agonist and specifically activate genes responsive to RXR homodimers. In addition exposure of frog larvae to methoprenic acid caused developmental deformities consistent with those that have been observed in recent years in wild populations and consistent with those caused by exposure to retinoic acid under laboratory conditions. These observations indicate that methoprenic acid functions as an RXR agonist, and that this activity could contribute to the occurrence of amphibians deformities documented in the environment.

17.3.2 Hormone Receptor Antagonists

While the estrogen receptor appears somewhat unique among vertebrate nuclear hormone receptors in its promiscuity toward receptor agonists, many nuclear hormone receptors have been shown to be susceptible to chemical antagonism. Receptor antagonists are defined as *chemicals that bind to a hormone receptor but do not activate the receptor*. Rather, these chemicals inhibit receptor activity by preventing the endogenous hormone from binding to and activating the receptor.

Estrogen Receptor. Chemicals often bind to the estrogen receptor and function as mixed agonists/antagonists (discussed below). For example, the drug tamoxifen functions as an estrogen receptor antagonists in reproductive tissue but functions as an agonist with respect to the preservation of bone mineral density and reducing serum cholesterol concentrations. Accordingly tamoxifen can function as a prophylactic against the growth of estrogen-responsive breast cancers and osteoporosis via two different mechanisms (estrogen receptor antagonism and agonism, respectively). Other drugs that bind to the estrogen receptor as an antagonist or mixed agonist/antagonist include raloxifene,

ICI 164,384, and toremifene. Environmental estrogen receptor antagonists include some phytochemicals (i.e., flavonoids) and PCBs (i.e., 3, 3′, 4, 4′-tetrachlorobiphenyl). Consequences of estrogen receptor antagonism are typically considered de-feminization (loss of female traits). In laboratory animal studies, estrogen receptor antagonists have been shown in females to disrupt estrous cycles, impair fertility, increase preimplantation loss, and cause embryolethality.

Androgen Receptor. Chemicals that bind to the androgen receptor in an antagonistic fashion include the pharmaceuticals spironolactone, cimetidine, cyperoterone acetate, and hydroxyflutamide. Environmental chemicals that have been shown to act as androgen receptor antagonists include the metabolites of the agricultural fungicide vinclozolin, the DDT metabolite *p*, *p*′-DDE, some hydroxylated PCBs, and the organophosphate insecticide fenitrothion. The consequence of androgen receptor antagonism is typically considered demasculinization (loss of male traits). Demasculinizing effects of antiandrogens in laboratory animal studies have included reductions in the size of the ventral prostate and seminal vesicle weights along with deformities of the penis.

Glucocorticoid Receptor. Some drugs (i.e., mifepristone) elicit antagonistic activity toward the glucocorticoid receptor. This property has been associated with adverse side effects of some drugs and also has been capitalized upon therapeutically for the modulation of the glucocorticoid receptor. Antiglucocorticoids typically are steroidal compounds that are capable of binding to the receptors but are relatively ineffective in activating the receptor. As such, these compounds are typically mixed agonists/antagonists (see below). Glucocorticoid receptor antagonists can adversely affect growth, development, and other glucocorticoid-regulated processes (Table 17.1). Little is known of the ability of environmental chemicals to function as glucocorticoid receptor antagonists.

Mixed Agonists/Antagonists. Chemicals often can function as either a receptor agonist or antagonist depending on the level of endogenous hormone. A weak agonist may bind to a receptor and stimulate some low-level receptor-mediated activity in the absence of the endogenous hormone. However, in the presence of the hormone, binding of the xenobiotic to the receptor may prevent binding of the endogenous hormone, and if the xenobiotic is a much weaker activator of receptor-mediated activity, then the net effect is loss of activity. Thus, in the presence of the endogenous hormone, the xenobiotic functions as a receptor antagonist. Whether a weak xeno-agonist functions as an agonist or antagonist depends on (1) the concentration of the xeno-agonist, (2) the binding affinity of the xeno-agonist to the receptor, (3) the concentration of the endogenous hormone to the receptor, and (4) the binding affinity of the endogenous hormone to the receptor. These compounds are classified mixed agonists/antagonists.

17.3.3 Organizational versus Activational Effects of Endocrine Toxicants

Effects of receptor agonists or antagonists on endocrine related processes are often described as being either organizational or activational. An organizational effect of an endocrine toxicant is one that typically results from neonatal or prenatal exposure during which time hormones are directing various irreversible aspects of development.

Accordingly the disrupting effect of the toxicant also is irreversible. These organizational effects may be evident only later in life during maturation or reproduction. Neonatal exposure to DES resulting in proliferation of epithelial cells of the reproductive tract at reproductive maturity is an example of an organizational effect of an endocrine toxicant. Organizational effects of endocrine toxicants have been of great concern to toxicologists and are the most difficult type of toxicity to diagnose owing to the temporal separation between exposure and effect.

An activational effect of an endocrine toxicant occurs in the same time frame as the exposure and is the consequence of the toxicant disrupting the immediate role of a hormone in some physiological process. Activational effects are reversible following cessation of exposure to the toxicant. For example, androgens contribute to maintenance of the prostate gland in the adult male. Exposure of adult males to an antiandrogen can result in a decrease in prostate size. Cessation of exposure to the antiandrogen then results in restoration of the prostate gland to its normal size.

17.3.4 Inhibitors of Hormone Synthesis

Endocrine toxicants can elicit antihormone activity by lowering levels of endogenous hormone in the body. With steroid hormones, chemicals typically elicit this effect by inhibiting enzymes necessary for synthesis of the hormone. For example, the cytochrome P450 enzyme CYP19 is responsible for the aromatization of testosterone to form 17β-estradiol. CYP19 inhibitors such as fadrozol, anastrozole, and letrozole, can lower endogenous 17β-estradiol levels resulting in de-feminization. Cytochrome P450s enzymes also are critical to various hydroxylation reactions that contribute to the synthesis of androgens and other steroid hormones and inhibition of these enzymes can result in a variety of antisteroid hormone effects. For example, the agricultural and medicinal fungicides propiconazole, ketoconazole, and fenarimol are capable of inhibiting P450 enzymes and reducing synthesis and circulating levels of testosterone and other steroid hormones. Toxicological consequences of the lowering of endogenous steroid hormone levels are typically comparable to those effects elicited by antagonists of the hormone's receptor.

17.3.5 Inducers of Hormone Clearance

In most species, steroid and thyroid hormones are inactivated and cleared from the body by the same biotransformation processes that are involved in chemical detoxification (see Chapter 7). Predominant among the hormone biotransformation processes in vertebrates are hydroxylation, glucuronic acid conjugation, and sulfate conjugation. The thyroid hormones T_3 and T_4 are inactivated and cleared following sulfate and glucuronic acid conjugation, respectively. The glucuronosyl transferase enzymes that are responsible for the elimination of T_4 are induced following exposure to phenobarbital-type inducers and Ah receptor ligands (see Chapter 9). Thus exposure to chemicals such as some dioxins and PCBs can result in enhanced clearance of thyroid hormone resulting in low circulating thyroid hormone levels. The resulting hypothyroid state can result in a variety of pathological conditions. In newborn infants, hypothyroidism is associated with cretinism. This organizational syndrome is characterized by mental retardation, short stature, and various neurological abnormalities. In children,

hypothyroidism can cause delayed growth and mental development while advancing the onset of puberty in adolescents. Hypothyroidism in adults results in various activational abnormalities including impaired cardiovascular, pulmonary, intestinal, and renal function. Chronic fatigue, lethargy, and difficulty in concentration are also associated with thypothyroidism in adults.

Increased clearance of steroid hormones due to induction of hepatic biotransformation enzymes following chemical exposure often has been cited as a possible mechanism by which toxicants could lower circulating testosterone or 17β-estradiol levels. While enhanced clearance of sex steroids has been demonstrated following chemical exposure and induction of hepatic biotransformation enzymes, elegant feedback control mechanisms tend to ensure that more hormone is produced and homeostasis is maintained (Figure 17.2). Enhanced clearance of sex steroids can contribute to endocrine disruption if the toxicity also results in impaired hormone synthesis (i.e., gonadal toxicity or interference with the feedback control of hormone synthesis). 2,3,7,8-Tetrachlorodibenzodioxin appears to lower circulating sex steroid levels via this dual effect.

17.3.6 Hormone Displacement from Binding Proteins

Steroid and thyroid hormones are typically distributed throughout the body while bound to serum-binding proteins such as sex hormone-binding globulin, corticosteroid-binding globulin, thyroxine-binding globulin (transthyretin), and albumin. Most steroid and thyroid hormones (>95%) are present in the blood reversibly bound to proteins. This bound hormone is not available for cell entry where it may interact with nuclear receptors or undergo inactivation/elimination reactions. Rather, the bound hormone serves as a reservoir from which hormone can be liberated (free hormone) for cell entry.

Some xenobiotics can compete with hormones for binding to the blood proteins. As a result the circulating hormone reservoir can be depleted and free hormone becomes limited. A variety of phenolic compounds, including hydroxylated metabolites of polychlorinated biphenyls (PCBs), chlorophenols, chlorophenoxy acids, and nitrophenols, have been shown to interfere with thyroxine binding to thyroxine-binding globulin during in vitro experiments. In some instances compounds that displace thyroxine from the binding protein also have been shown to decrease circulating thyroxine levels in exposed animal models or in humans. In vitro experiments also have revealed that testosterone and 17β-estradiol can be displaced from sex hormone-binding globulin by some chemicals such as 4-nonylphenol, 4-tert-octylphenol, bisphenol A, *O*-hydroxybiphenyl, and pyrethroid insecticides. However, it is not clear whether these chemicals would significantly displace sex steroids from the binding globulin at concentrations typically measured in human blood.

17.4 INCIDENTS OF ENDOCRINE TOXICITY

17.4.1 Organizational Toxicity

In utero exposure to estrogens or antiandrogens has been shown, in animal models, to elicit a variety of organizational effects associated with development of the reproductive system. The best-described example of the organizational effects of a drug

administered to humans involves the synthetic estrogen DES. As discussed previously, DES was prescribed to over two million pregnant women in the United States between the 1940s and 1960s to prevent miscarriage. Offspring exposed to DES during fetal development experienced a variety of problems upon attainment of sexual maturity. DES daughters experience a significantly increased risk of clear cell adenocarcinoma of the vagina and cervix. DES daughters have increased risk of a variety of reproductive disorders including structural abnormalities of the reproductive tract, infertility, ectopic pregnancy, miscarriage, and pre-term delivery.

Less is known of the risks faced by males exposed to DES during fetal development. Animal studies have revealed that male rodents exposed to DES have increased incidence of prostatic metaplasia. Epidemiological studies of DES sons have suggested increased risk of various testicular abnormalities including epididymal cysts, testicular varicoceles, and undescended testis. Hyperplasia and metaplasia of the prostatic ducts in DES sons also have been reported.

The effects elicited by fetal exposure to DES appear to be largely the consequence of the estrogenic activity of this drug. Estrogens orchestrate organizational events during fetal development that promote female reproductive tract development. Excess estrogen exposure resulting from DES treatment of either female or male fetuses resulted in permanent alterations, many of which became evident only upon attainment of reproductive maturity.

Organizational effects on reproductive development resulting from perinatal exposure to endocrine toxicants of environmental origin also have been reported to occur. In 1973 a fire retardant containing polybrominated biphenyls (PBBs) was mistakenly added to cattle feed in Michigan. An estimated 4000 people subsequently were exposed to the PBBs by consuming dairy products derived from these cattle. PBBs are long-lived chemicals that are stored in the fat of exposed individuals. PBBs have been reported to elicit endocrine toxicity-like symptoms in animal models consistent with hypothyroidism. For example, offspring from maternal rats provided PBBs during gestation and lactation showed signs of neurological deficit and growth retardation. Daughters of mothers that were exposed to PBBs during the Michigan incident were monitored for possible adverse effects on the female reproductive system. The initiation of menarche (menstruation) among these daughters correlated with the likely severity of PBB exposure. The most highly exposed daughters began menstruating approximately 1 year ahead of females that were less severely exposed. Early initiation of menarche is consistent with precocious puberty associated with hypothyroidism. The initiation of menarche also is under the regulation of 17β-estradiol and early initiation of menarche may reflect an estrogen-type organizational effect of the PBBs during perinatal exposure.

17.4.2 Activational Toxicity

Estrogenic Pharmaceuticals. Administration of estrogenic pharmaceuticals to children or adults can result in a variety of abnormalities associated largely with secondary sex characteristics that are reversible upon cessation of drug treatment.

Gynecomastia, the development of breast tissue in males, is often the consequence of perturbations in the normal androgen/estrogen ratio. As discussed earlier in this chapter, prolonged administration of drugs with estrogenic or antiandrogenic activity can cause gynecomastia. Gynecomastia had been reported in the medical literature

to occur as a result of frequent intercourse when an estrogen-containing cream was used as a vaginal lubricant and among morticans who applied estrogen-containing skin creams to corpses without the use of gloves.

Similar to gynecomastia in adult males, activational toxicity from estrogenic drugs has been reported to cause pseudoprecocious puberty in children. Pseudoprecocious puberty is characterized by the development of some indicators of puberty (pubic or facial hair, morphological changes in sex organs, breast development, etc.) in preadolescent individuals. An outbreak of pseudoprecocious puberty was reported among a group of children ranging in age from 4 months to 2 years of age following application of a skin cream to treat dermatitis. Symptoms included pigmentation of the nipples, breast development, the presence of pubic hair, and vaginal discharge and bleeding among the females. Breast development also was reported in prepubertal boys following use of an estrogen-containing hair cream. These reports highlight the fact that dermal exposure can be adequate to attain a sufficient dose of endocrine-active compound to elicit adverse responses. In all of these cases the symptoms of endocrine toxicity resolved following cessation of exposure to the causative agent.

Environmental Estrogens. Thelarche is defined as the development of breast tissue in preadolescent females (typically <8 years of age). Since 1979 physicians have monitored an epidemic level of thelarche on the island of Puerto Rico. The cause of thelarche in Puerto Rico is not known; however, evidence strongly implicates exposure to endocrine-disrupting agents. Analyses of blood samples from thelarche and nonthelarche children for environmental chemicals with known estrogenic activity revealed that 68% of the thelarche children contained significantly high levels of several types of phthalate esters. Only a single nonthelarche child contained a significant amount of phthalate ester and only one type of phthalate ester was found in this individual. Phthalate esters are used as plasticizers and are ubiquitous environmental contaminants. They have been shown to cause a variety of endocrine-related effects in animal models and some phthalate esters have been shown to be estrogenic in vitro. The association between phthalate ester exposure and the high incidence of thelarche in Puerto Rico does not establish causality but has generated concern that environmental agents are responsible for this condition.

Kepone (chlordecone) is an organochlorine insecticide (Figure 17.5) that was manufactured in Hopewell, Virginia, from the mid-1960s to 1975. In 1975 the Center for Disease Control determined that employees of the manufacturing facility and other residents of Hopewell, totaling over 200 individuals, had been significantly contaminated with this insecticide. Exposed individuals reported a variety of symptoms. Foremost, among the symptoms of "Kepone sickness" were neurological disorders presenting as tremors, weight loss, and nervousness. However, subsequent evaluations revealed that males exposed to Kepone also experienced testicular dysfunction that was characteristic of estrogen exposure. Later laboratory studies demonstrated that Kepone was an estrogen receptor agonist, which could explain its adverse effects on the male reproductive system.

17.4.3 Hypothyroidism

Hypothyroidism describes the clinical state arising from a deficiency in thyroid hormone. Toxicity resulting in hypothyroidism is manifested at several organ systems as

Table 17.4 Clinical Manifestations of Hypothyroidism

Organ System	Manifestation
Skin	Puffy appearance, dry, course, yellow-tinted skin brittle nails, wound healing slowed, hair loss
Cardiovascular	Enlarged heart, changes in electrocardiographs
Respiratory	Maximal breathing capacity reduced, obstructive sleep apnea, fluid accumulation in the pleural cavity
Digestive	Reduced appetite with modest weight gain
Muscle	Stiffness, aching
Nervous	Slowing of intellectual functions, lethargy, headaches

described in Table 17.4, and individual effects may be misdiagnosed as organ-specific toxicity. Hypothyroidism can result from various causes other than chemical toxicity including diseases of the hypothalamic–pituitary–thyroidal axis, iodine deficiency, and heritable defects in thyroid hormone production. Chemical agents that have historically been recognized for their ability to cause hypothyroidism include phenylbutazone, resorcinol, lithium, and para-aminosalicylic acid.

Disruptions in thyroid hormone levels can occur through chemical-induced increases in the metabolic inactivation and elimination of the hormone. Chemicals that are capable of increasing the metabolic clearance of thyroid hormone include the polycyclic halogenated hydrocarbons (i.e., dioxins, furans, polychlorinated biphenyls, polybrominated biphenyls). A study reported in the *New England Journal of Medicine* suggested that environmental or occupational exposure to such chemicals can result in hypothyroidism in humans. The study consisted of a comparison of thyroid status in workers who were occupationally exposed to polybrominated biphenyls as compared to workers who were not exposed to any polyhalogenated hydrocarbons. Four of 35 exposed workers and none of 89 unexposed workers exhibited signs of hypothyroidism that included increased plasma levels of thyrotropin and decreased plasma levels of thyroxine. Thyrotropin is secreted by the pituitary gland and stimulates the thyroid gland to produce thyroxine (see Figure 17.1). The increase in thyrotropin and decrease in thyroxine is consistent with hypothyroidism caused by increased clearance of the thyroxine. As discussed earlier in this chapter, perinatal exposure to PBBs during the Michigan milk contamination also produced symptoms characteristics of hypothyroidism.

17.5 CONCLUSION

The endocrine system possesses many targets at which toxicants can elicit either reversible or permanent effects on an individual. Effects of chemicals on endocrine-regulated processes such as development, maturation, growth, and reproduction have been well documented in both laboratory and epidemiological studies. Less is know of the potential effects of endocrine toxicants on more generalized endocrine-regulated processes such as bone maintenance, general organ function, and metabolism. The US Environmental Protection Agency has been mandated by the US Congress to develop and implement a program for the screening and testing of chemicals for endocrine-disrupting toxicity. At this writing, the EPA is in the process of developing such a

program that will focus on the effects of chemicals on the androgen/estrogen and thyroid hormone regulated processes. Once implemented, this required testing will greatly expand our knowledge of the extent to which humans are exposed to chemicals that interfere with processes regulated by these hormones. However, it is important to recognize that chemicals have the potential ability to interfere with other hormone cascades, including those involving mineralcorticoids, glucocorticoids, retinoids, and perhaps some peptide hormones. Research is needed to increase our understanding of the susceptibility of endocrine signaling pathways involving these hormones to chemical toxicity and, ultimately, to our establishing chemical exposure limits that include these considerations.

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CHAPTER 18

Respiratory Toxicity

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18.1 INTRODUCTION

Pulmonary diseases caused by agents in the environment have been known for centuries and have been associated with occupations such as stone quarrying, coal mining, and textiles. The problem is more complex and widespread today because new agents are constantly being added to the environment. They include all types of inhalant toxicants, gases, vapors, fumes, aerosols, organic and inorganic particulates, and mixtures of any or all of these. Gasoline additives and exhaust particles, pesticides, plastics, solvents, deodorant and cosmetic sprays, and construction materials are all included. Table 18.1 lists some of the more important industrial lung toxicants, the exposure sources, and associated injuries.

18.1.1 Anatomy

Air enters the respiratory systems of mammals through the nose or mouth, some, including humans, utilizing both while others being obligatory nose breathers. Inhaled air then passes into the proximal airways, the trachea, and the main bronchi to each lung. The main bronchi then subdivide several times into numerous bronchi, finally into terminal bronchioles and respiratory bronchioles, ultimately ending in alveolar ducts and alveoli (Figure 18.1).

18.1.2 Cell Types

As shown in Table 18.2, there are many different cell types in the respiratory system with considerable variation in both structure and function from the nasal epithelium to the alveoli. The various cell types of the airway epithelium are shown in Figure 18.2.

18.1.3 Function

The nasal passages have an olfactory function, but with regard to inhaled toxicants they have primarily a defensive function and form the initial defensive barrier against inhaled

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Table 18.1 Some Important Industrial Lung Toxicants and Associated Injury

Toxicant	Source	Damage
Aluminum dust	Ceramics, paints, fireworks, electrical goods	Fibrosis
Ammonia	Manufacture of fertilizers, explosives, ammonia	Irritation
Arsenic	Manufacture of pesticides, glass, pigments, alloys	Lung cancer, bronchitis
Asbestos	Mining, construction, shipbuilding	Asbestosis, lung cancer
Beryllium	Ore extraction, ceramics, alloys	Fibrosis, lung cancer
Cadmium oxide	Welding, smelting, manufacture of electronics, alloys, pigments	Emphysema
Chlorine	Manufacture of pulp and paper, plastics, chlorinated chemicals	Irritation
Chromium	Manufacture of Cr compounds, paint pigments	Lung cancer
Coal dust	Coal mining	Fibrosis
Hydrogen fluoride	Manufacture of chemicals, plastics, photographic film, solvents	Irritation, edema
Iron oxides	Welding, steel manufacturing, mining, foundry work	Fibrosis
Nickel	Nickel extraction and smelting, electroplating	Nasal cancer lung cancer, edema
Nitrogen oxides	Welding, explosive manufacturing	Emphysema
Ozone	Welding, bleaching, deodorizing	Emphysema
Phosgene	Production of pesticides, plastics	Edema
Silica	Mining, quarrying, farming	Fibrosis (silicosis)
Sulfur dioxide	Bleaching, refrigeration, fumigation coal combustion	Irritation
Talc	Rubber industry, cosmetics	Fibrosis
Tetrachloroethylene	Dry cleaning, metal degreasing	Edema

toxicants. Since the nasal epithelium contains relatively high levels of xenobiotic-metabolizing enzymes such as CYP and FMO isoforms, it can function as a detoxification site. However, as these enzymes, particularly CYP, may also activate toxicants, the nose is also a site for toxicant-induced lesions.

The trachea and bronchi likewise have a protective function. Mucous and serous cells secrete fluids that together comprise the mucus, which is moved toward the pharynx by the cilia of the ciliated cells. The movement of mucus serves to move entrapped particles toward the pharynx where they are eliminated by swallowing or expectoration. The mucus may also have other protective functions, protecting the epithelial cells by free radical scavenging and antioxidant properties. The Clara cells are known to contain high concentrations of xenobiotic metabolizing enzymes.

The principal function of the lungs is gas exchange, providing O_2 to the tissues and removing CO_2. This gas exchange takes place in the alveoli. Because the lung has a large surface area and exchanges a significant volume of air (100,000–20,000 L/day

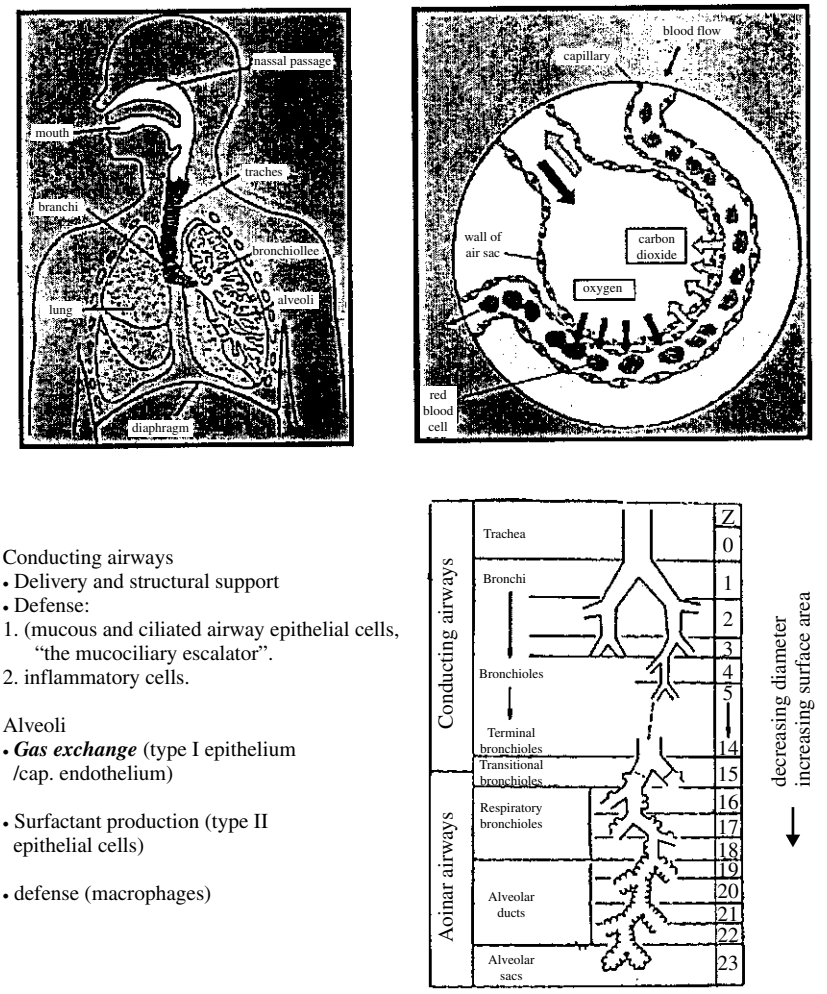

Figure 18.1 Structure and function of the respiratory system.

Table 18.2 Cell Types of the Respiratory System

Nasal	Stratified squamous to mucociliated epithelium with olfactory cells
Tracheo-bronchial region	Mucociliated epithelium (ciliated, mucous cells, basal cells); smooth muscle cells; fibroblasts; neuroendocrine cells; immune cells
Bronchioles	Mucociliated epithelium with Clara cells in distal bronchioles and alveolar ducts
Alveoli	Type I and type II epithelium, alveolar macrophages

for the average adult), the lung is the major interface between an organism the environment and any toxicants present in the air. It is also significant, from an efficiency point of view, that the entire cardiac output goes to the lungs.

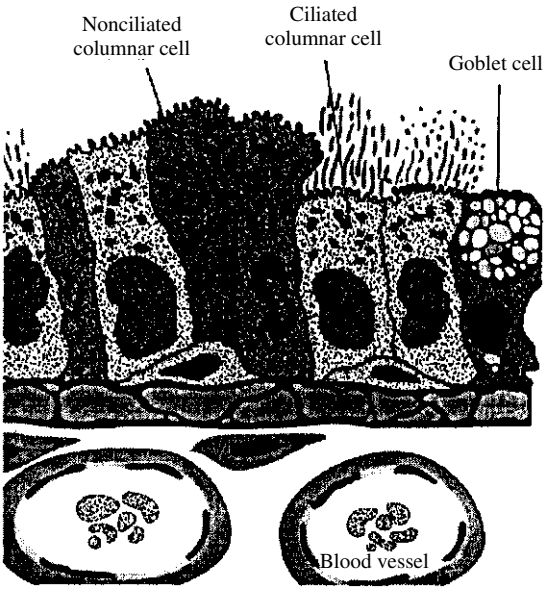

Figure 18.2 Cell types of the airway epithelium.

18.2 SUSCEPTIBILITY OF THE RESPIRATORY SYSTEM

18.2.1 Nasal

The nasal epithelia are the first point of contact for respiratory toxicants. Because they contain xenobiotic metabolizing enzymes, they are susceptible to toxic effects caused by reactive intermediates.

18.2.2 Lung

In addition to being in direct contact with airborne toxicants, the entire body blood volume passes through the lung one to five times a minute, exposing the lung to toxicants and drugs within the systemic circulation. Thus the possibility of damage from both inhaled and circulating agents is enormous. As with the liver and kidney, the lungs possess significant levels of many xenobiotic metabolizing enzymes and thus can play a large role in the activation and detoxication of exogenous chemicals.

18.3 TYPES OF TOXIC RESPONSE

Although many different agents may damage the lung, the patterns of cellular injury and repair are relatively constant, and most fall into one or more of the categories described below.

18.3.1 Irritation

Perhaps one of the most obvious and familiar chemical effects is irritation caused by volatile compounds such as ammonia or chlorine gas. Such irritation, especially

if severe or persistent, results in constriction of the airways. Edema and secondary infection frequently follow severe or prolonged irritation. Such damage is known to result from exposure to agents such as ozone, nitrogen oxides, and phosgene.

18.3.2 Cell Necrosis

Severe damage to the cells lining the airways can result in increased cell permeability, followed by cell death.

18.3.3 Fibrosis

Fibrosis, or formation of collagenous tissue, was perhaps one of the earliest recognized forms of occupational diseases. *Silicosis*, resulting from inhalation of silica (SiO_2), is thought to involve first the uptake of the particles by macrophages and lysosomal incorporation, followed by rupture of the lysosomal membrane and release of lysosomal enzymes into the cytoplasm of the macrophages. Thus the macrophage is digested by its own enzymes. After lysis, the free silica is released to be ingested by fresh macrophages, and the cycle continues. It is also thought that the damaged macrophages release chemicals that are instrumental in initiating the collagen formation in the lung. Fibrosis may become massive and impair the respiratory function of the lung significantly. *Asbestosis* was recognized as long ago as 1907; however, the magnitude of the risk has become apparent only recently, primarily due to the increased incidence of lung cancer among asbestosis sufferers, especially those who are also cigarette smokers. Both silicosis and asbestosis are thought to be premalignant conditions.

18.3.4 Emphysema

Emphysema is characterized by an enlargement of the airspaces with the destruction of the gas-exchange surface area. The loss of tissue and air-trapping capacity results in a distended lung that no longer effectively exchanges O_2 and CO_2. Although cigarette smoking is the major cause of emphysema, other toxicants can also cause this condition.

18.3.5 Allergic Responses

Numerous agents, including microorganisms, spores, dust, and chemicals, are known to elicit allergic responses resulting in constriction of the airways. Several diverse examples are farmer's lung from the spores of a mold that grows on damp hay, maple bark stripper's disease from spores of a fungus growing on maple trees, cheese washer's lung from penicillin spores, and mushroom picker's lung from the mushroom spores. Byssinosis comes from the inhalation of cotton, flax, or hemp dusts. This condition, however, does not seem to result from bacterial or fungal exposure but from an apparent toxicant or allergen associated with the plant dusts.

18.3.6 Cancer

Perhaps the most severe response of the lung to injury is cancer, with the primary cause of lung cancer being cigarette smoking. Cigarette smoke contains many known

carcinogens as well as lung irritants. Many of the polycyclic aromatic hydrocarbons, such as benzo(*a*)pyrene, can be metabolized in the lung by pulmonary P450 enzymes to reactive metabolites capable of initiating cancer. In addition cigarette smoke contains numerous compounds that can act as tumor promoters. Asbestos is associated with two forms of cancer—lung cancer and malignant mesothelioma, a tumor of the cells covering the surface of the lung and the adjacent body wall.

18.3.7 Mediators of Toxic Responses

Most of the toxic responses summarized above involve a relatively small number of biochemical events related to oxidative injury, signaling pathways, and genotoxicity.

Reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2$) and hydroxyl radical ($\cdot OH$) are balanced by antioxidant enzymes and radical scavengers. Imbalance favoring ROS generation leads to lipid peroxidation, with resultant membrane damage, and DNA damage (genotoxicity).

Changes in the concentration of growth factor and/or cytokine signaling molecules may lead to inflammation, proliferative responses, or apoptosis.

18.4 EXAMPLES OF LUNG TOXICANTS REQUIRING ACTIVATION

18.4.1 Introduction

The activation of pulmonary toxicants falls into three main categories or mechanisms, depending either on the site of formation of the activated compound or on the nature of the reactive intermediate.

1. The parent compound may be activated in the liver, with the reactive metabolite then transported by the circulation to the lung. As would be expected, the activated compounds may lead to covalent binding and damage to both liver and lung tissue.
2. A toxicant entering the lung, either from inhaled air or the circulatory system, may be metabolized to the ultimate toxic compound directly within the lung itself. Although the total concentration of P450 is less in the lung than in the liver, the concentration varies considerably in the different cell types, with the highest concentration being found in the nonciliated bronchiolar epithelial (Clara) cells of the terminal bronchioles. Because of this, the Clara cells are often a primary target for the effects of activated chemicals.
3. Another means of metabolic activation is the cyclic reduction/oxidation of the parent compound, resulting in high rates of consumption of NADPH and production of superoxide anion. Either the depletion of NADPH and/or the formation of reactive oxygen radicals could lead to cellular injury.

The following three chemicals serve to illustrate these three mechanisms of activation.

18.4.2 Monocrotaline

The pyrrolizidine alkaloids, found in the genus *Senecio* and a number of other plant genera, are plant toxins of environmental interest that have been implicated in a number

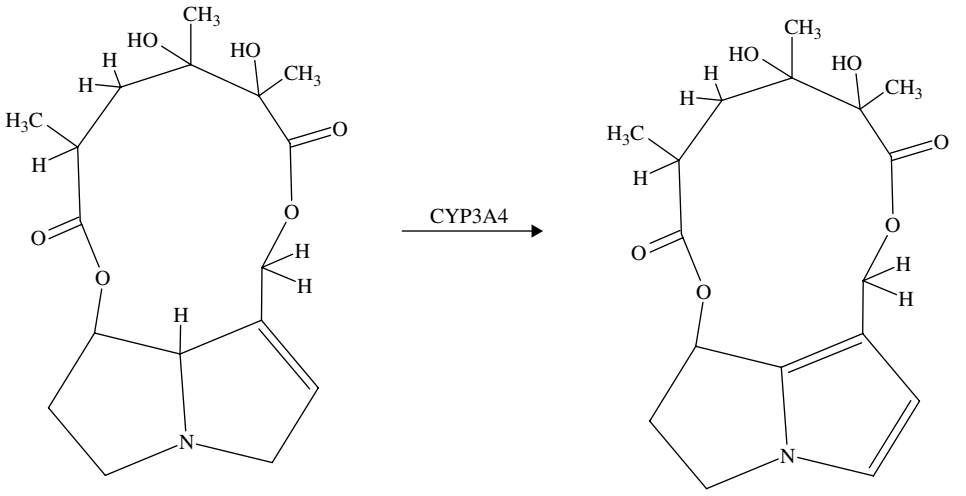

Figure 18.3 Structure and activation of monocrotaline.

of livestock and human poisonings. Grazing animals may be poisoned by feeding on pyrrolizidine alkaloid-containing pastures, and human exposure may occur through consumption of herbal teas and contaminated grains and milk. The chemical structure for monocrotaline is shown in Figure 18.3.

Monocrotaline, found in the leaves and seeds of the plant *Crotalaria spectabilis*, has been the most extensively studied of the pyrrolizidine alkaloids. When monocrotaline is given to rats and other animals at high doses, a pronounced liver injury occurs, and animals usually die of acute effects, presumably liver failure. Lower doses, however, that are only mildly hepatotoxic, result in lung injury that is associated with pulmonary hypertension and usually death in several weeks. It is thought that activation of monocrotaline to its dehydro metabolite occurs in the liver and is a reductive reaction mediated by cytochrome P450 3A4 (Figure 18.3). Even though monocrotaline acts as a pneumotoxicant, several lines of evidence indicate that the lung is incapable of activating monocrotaline or can only do so to a limited extent. Furthermore the main site of pulmonary injury occurs in the endothelial cells, a target site consistent with a reactive metabolite being absorbed from the circulatory system.

18.4.3 Ipomeanol

One of the best-known examples of a toxic compound being activated in the lung is 4-ipomeanol (Figure 18.4). This naturally occurring furan is produced by the mold *Fusarium solani* that infects sweet potatoes. Lung edema in cattle is known to be associated with the ingestion of mold-damaged sweet potatoes. A similar pulmonary lesion can be produced in a number of species regardless of the route of administration. Pulmonary injury by 4-ipomeanol is caused, not by the parent compound, but by a highly reactive alkylating metabolite produced in the lung by lung-specific P450 isozymes. In addition these isozymes are highly concentrated in the Clara cells, which are most affected by 4-ipomeanol toxicity. Although the reactive metabolite has not been identified unambiguously, considerable data suggest a reactive epoxide.

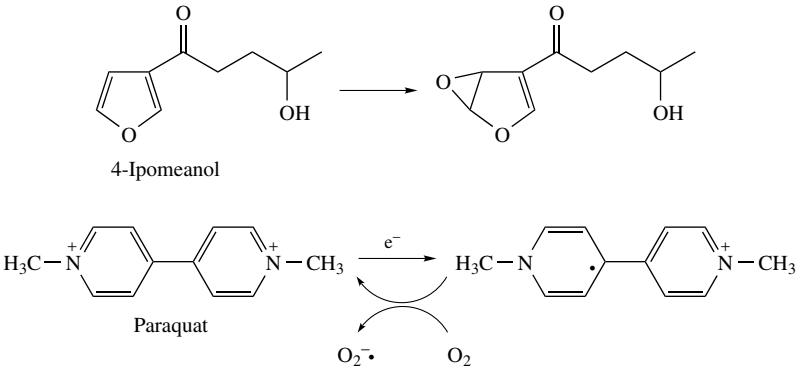

Figure 18.4 Structure and activation of ipomeanol and paraquat.

Other toxic lung furans, such as the atmospheric contaminants 2-methylfuran and 3-methylfuran, may exert their toxicity through the formation of reactive metabolites, probably reactive aldehydes.

18.4.4 Paraquat

Systemic administration of compounds such as the herbicide paraquat (Figure 18.4), bleomycin (a cancer therapeutic agent), and nitroflurantoin (an antibiotic used for urinary tract infections) initiate a progression of degenerative and potentially lethal lesions in the lung by a mechanism known as *redox cycling*. These compounds are reduced by cytochrome P450 reductase and NADPH, forming a free radical. Although the free radical could potentially react with tissue macromolecules, one molecule of oxygen is reduced to superoxide that can then be converted to other toxic oxygen species. These reactive compounds may cause peroxidation of cellular membranes. The specific toxicity of paraquat to the lung results from the uptake of this compound by the polyamine transport system in the lung as well as from the high pulmonary oxygen tension. Nitroflurantoin, however, is not actively accumulated in the lung, and its tissue specificity probably results from the high pulmonary oxygen tension.

18.5 DEFENSE MECHANISMS

There are two important defense mechanisms against inhaled particles. The first of these involves the mucociliary escalator and consists of the trapping of particles in mucus followed by the upward movement of the mucus brought about by the upward beating of cilia on the airway epithelial airway cells. The material is then either swallowed or expectorated. The second mechanism is macrophage mediated. Macrophages engulf particles and either deposit them on the mucociliary escalator or enter the lymphatic system.

Toxicants may also be detoxified by xenobiotic-metabolizing enzymes such as cytochrome P450 or the flavin-containing monooxygenase. It should be borne in mind, however, that these enzymes may also activate toxicants to more reactive, and potentially more toxic, metabolites.

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