19.1 DEFINITION OF A RECEPTOR

Although the concept of a receptor as a selective locus of action was pioneered more than a century ago, receptor theory remains a critical concept in toxicology and in many other branches of biology. In one sense, receptors may be considered as macromolecules that bind small molecules (commonly termed ligands) with high affinity and thereby initiate a characteristic biochemical effect. In some fields of biology, the term receptor has specific constraints. For example, in a cell biological context, the term refers to macromolecules (intracellular or cell surface) that recognize endogenous ligands; these ligands may be small (e.g., neurotransmitters, hormones, autacoids) or large (e.g., proteins involved in protein sorting or in intracellular scaffolding). In toxicology and pharmacology, the term “receptor” is often used to refer to the high-affinity binding site that initiates the functional change(s) induced by a xenobiotic (e.g., toxicity). It is important to note that the interaction of two macromolecules can be studied in much the same way as a ligand and a single macromolecule, and such macromolecular interactions have great importance to (a) biological systems in general and (b) toxicology in particular. The use of fundamental structural precepts (both steric and electrostatic) to understand the “fit” between receptor and ligand has become more feasible during the past few years based on advances in molecular biology and in computational chemistry and molecular modeling.

19.1.1 A Brief History of the Concept of Receptors

The concept of receptors is credited to the independent work of Paul Ehrlich (1845–1915) and J. N. Langley (1852–1926). With Ehrlich, the concept appeared to originate from his immunological studies on antibody–antigen interactions. Based on the high degree of specificity of antibodies for antigens, Ehrlich postulated the existence of stereospecific, complementary sites on the two molecules. Similar
reasoning led Emil Fisher to formulate the idea of a lock-and-key fit between enzymes and their substrates. In later studies with arsenicals to be used against syphilis and trypanosomes, Ehrlich observed that slight modification in chemical structure could dramatically affect the potency of a compound. This high degree of stereospecificity suggested to Ehrlich that his receptor theory was also relevant to drugs, and it also led to the concept of structure–activity relationships. Although this idea seems straightforward today, it was decades before physical evidence for this idea could be gleaned.

The physiological importance of receptors was shown by Langley. Studying the denervated frog neuromuscular junction, Langley observed that muscle contraction could be elicited when nicotine was applied to the denervated muscle and that this effect could be blocked by curare. He postulated the existence of “the receptive substance” that could bind both nicotine and curare; this was the first formulation of what is now called the neurotransmitter receptor. Langley also speculated that the formation of complexes between drug and receptive substance could be described lawfully through consideration of the relative concentration of a drug and its affinity for the receptive substance. This latter speculation predated the formal mathematical description of drug–receptor interactions according to mass action principles.

19.1.2 Toxicants and Drugs as Ligands for Receptors

The rationale for applying principles derived from the study of drug–receptor interactions to questions concerning possible toxicant–receptor interactions becomes clear when one considers that a drug is defined broadly as any chemical agent that affects living organisms; thus, a poison (toxicant) can be considered as a drug that has been administered at a dose level that results in detrimental effects, even death. Indeed, most clinically useful drugs have beneficial effects at one dose, yet become toxic (or even lethal) at higher doses. Clinical pharmacologists and toxicologists have quantified these observations in the term therapeutic index that can be defined:

\[
\text{Therapeutic index} = \frac{\text{TD}50}{\text{ED}50}
\]

The TD50 is the dose of a drug that would be toxic (or lethal) to one-half of a given population; the ED50 (ED for effective dose) is the dose that produces the beneficial effect in one-half of a comparable population. The therapeutic index is large for drugs that cause toxicity only at doses much higher than needed for therapy, and it is small for drugs that have a narrow margin of safety.

Although drugs are of considerable interest to toxicologists, other chemical agents are of at least equal importance. These include agricultural and industrial chemicals (e.g., herbicides, insecticides, chemical wastes, industrial solvents, etc.), as well as environmental contaminants. Whatever the source of a xenobiotic—drug or pollutant—its interaction with receptors can be described by the same general principles.

19.1.3 Is There a Normal Function for Toxicant Receptors?

As noted, a toxicant or drug receptor may be defined loosely as “a macromolecule with which a drug (or toxicant) interacts with high affinity to produce its character-
istic effect.” Inherent in this is the fact that binding of the toxicant (or drug) to the receptor causes a specific and predictable biological response. The obvious question, then, is whether the receptor of interest is one to which endogenous ligands bind or, rather, an opportunistic site for which the toxicant has affinity. Since this distinction is of general importance, several examples may be useful.

The pharmacological and toxicological properties of opium alkaloids (e.g., morphine and codeine) were known for centuries, and the framework provided by Langley, Ehrlich, Dale, and others led to the hypothesis that a specific receptor existed for these drugs. By the early 1970s, the approaches outlined in this chapter first were used to identify the “morphine receptor” (i.e., the target for the actions of morphine and other opiates). This was followed closely by evidence for three related opioid receptors (i.e., µ-, δ-, and κ-opioid receptors) that were the targets for endogenous neurotransmitter opioid peptides and for xenobiotics like morphine. Although the opioid system was one of the first systems in which direct evidence was presented for the binding of a foreign compound to a receptor, receptor methods are now widely used to study the mechanism of action of drugs and toxicants. Another similar example relates to the alkaloids derived from the “deadly nightshade” (Belladonna sp.). It is now known that atropine and related compounds bind to a subtype of receptor for the endogenous neurotransmitter acetylcholine. These are only two early and well-studied examples.

When an endogenous ligand is not known for a toxicant receptor, it raises the question whether the toxicant has found an adventitious site. An interesting historic example is the GABA<sub>A</sub> receptor, a schematic of which is shown in Figure 19.1. The GABA<sub>A</sub> receptor is one of the two major subclasses of recognition sites for the Figure 19.1. Schematic representation of GABA<sub>A</sub> receptor. This polymeric receptor regulates the influx of chloride, thus providing an inhibitory neuronal influence. It consists of five subunits of three major types (α, β, and γ), each of which has several subforms. There are differences in the subunit composition of the receptor, depending on where it is expressed. There are many sites at which xenobiotics may bind, thereby affecting the function of this receptor complex.
inhibitory neurotransmitter GABA (\(\gamma\)-aminobutyric acid). The GABA\(_{A}\) receptor belongs to, and is typical of, a superfamily of ligand-gated ion channels called ionotropic receptors (see Section 19.2). It is a polymer (in this case of five protein subunits each of ~50kD) arranged in a circle to form a channel. Each protein subunit is actually a string of amino acids which passes in and out of the cell membrane four times. The GABA\(_{A}\) receptor functions as an ion channel for the chloride anion. This channel remains closed until GABA binds to the recognition site.

The same portion of the receptor that recognizes GABA also binds certain toxicants such as the mushroom alkaloid muscimol. There is another site on the receptor that can bind a class of neuromodulators called neuroactive steroids. Several toxins, including picrotoxin, bind to the pore of this receptor. There is a third and distinct site on the GABA\(_{A}\) complex that was known for many years to bind a class of drugs called benzodiazepines (e.g., diazepam or Valium\(^8\)). For many years, the function of this second site was unknown, but it is now thought that a peptide called DBI (diazepam-binding inhibitor) is the endogenous ligand. As molecular genetics has provided information on the structure and function of receptors in the genome, it has resulted in better understanding of the receptor mechanisms of action of many toxicants.

19.1.4 Functional Biochemistry of Toxicant Receptors

From the viewpoint of biochemistry and neuroscience, “receptor” refers to a distinct class of molecules that have unique and characteristic functions and modes of action that involve endogenous ligands. Enzymes are, in one sense, receptors with respect to their substrates (and inhibitors, too). As will be demonstrated later in this chapter, the interaction of receptors with their ligands is similar conceptually to the interaction of enzymes and substrates or inhibitors. In practice, however, the interaction of ligands with receptors often differs from enzyme–substrate interactions in two qualitative, but important, ways. First, the affinity of ligands for receptors is usually two or more orders of magnitude higher than the affinity of enzymes for their substrates; second, enzymes act by biochemically altering substrate molecules, whereas receptors do not.

As noted above, toxicant receptors, per se, may serve a physiological role as receptors for a diverse group of endogenous ligands such as neurotransmitters, hormones, and autacoids. A common characteristic of these receptors is that they transduce chemical information of one type (the concentration of the ligand) into a variety of other secondary chemical events (including synthesis of second messengers, changes in ion flux or transport, and the like). For example, when the receptors are present on the extracellular surface of neurons, the ultimate effect of ligand binding is a change in the electrical excitability of the cell. In addition to post-synaptic receptors, cell surface receptors on neurons may be present on either presynaptic membranes or cell bodies. In these cases, they are termed auto-receptors and play a self-regulatory role (see Chapter 31 for specific examples).

Some ligands do not interact principally with cell surface receptors, but diffuse into cells and bind to intracellular receptors in the cytoplasm. For example, ligand binding to cytoplasmic steroid receptors initiates a process that is not well understood but that involves the movement of steroid-bound receptor into the cell nucleus, where the receptor molecule interacts with genomic material, resulting in alterations in gene expression and protein synthesis.
As will become clear, this chapter is focused on toxicants for which the receptor is a high-affinity recognition site of the type discussed in the previous paragraphs. It should be noted explicitly that other toxicants have “receptors,” but fall into more complex situations not appropriate for this chapter. For example, some toxicants inhibit enzymes, or are themselves enzymes. Such interesting compounds include the organophosphate and carbamate insecticides (acetylcholine esterase inhibitors) and diphtheria toxin (an enzyme).

19.1.5 Types of Interactions Between Toxicants and Receptors

Toxicant molecules that gain access to receptor sites in various tissues may then interact with those receptors in several ways. First, the interaction may mimic the endogenous ligand and cause agonist-like actions. Second, the molecule may bind to the receptor without causing resultant activation, thereby blocking access of an endogenous ligand (antagonist actions). Third, the toxicant may produce allosteric effects on receptors; for example, it has been shown that some toxicants, rather than binding to the same site as endogenous ligands, bind instead to an adjacent part of the macromolecule (see Section 19.2.2 and Figure 19.1 for examples). This interaction causes allosteric changes that affect the function of the complex, and sometimes the binding of the neurotransmitter itself. Finally, some macromolecules may have no endogenous ligands, yet bind specific toxicants that cause physiological changes via this interaction.

The four mechanisms listed above involve direct interaction of a toxicant with a receptor; in such cases, the toxicant–receptor interaction is likely to be involved in the mechanism of action. In many cases, toxicants may affect receptor function indirectly. For example, in the nervous system, decreases in synaptic transmission (by receptor blockade or damage to a neuron) may lead to increases in the number of receptors on the target neuron (so-called upregulation). This is often felt to be one of the compensatory mechanisms by which the nervous system responds to such perturbation. Conversely, increases in synaptic transmission (e.g., by long-term receptor activation) may lead to compensatory decreases (downregulation) in receptor number. The techniques by which such mechanisms are studied are described later in this chapter. It should be noted that the availability of molecular probes now permits evaluation not only of the characteristics of the binding sites, but also of the expression of the mRNA for the receptor(s) under study.

19.1.6 Goals and Definitions

The object of this chapter is to provide the foundation necessary for understanding the interactions of small molecules (ligands, be they toxicants or drugs) with receptors, and also the fundamentals of the techniques commonly employed for such analysis. It should be noted that the same theory is also useful for understanding the interaction of two large molecules, and the application of similar theory to such problems has increased as the importance of macromolecular interactions has been realized in recent years.

19.1.6a Definitions. There are several terms in common use that should be defined.
• **Affinity:** This is related to the "tenacity" by which a drug binds to its receptor, and it reflects the difference between the rates of association and dissociation of the ligand. Thus, a ligand that binds tightly to a receptor has a small equilibrium dissociation constant, or a large equilibrium association constant. A toxicant with high affinity for a receptor will bind to that receptor even at low concentrations, whereas binding of a low affinity toxicant will be evident only at higher concentrations.

• **Potency:** This term refers to the ability of a toxicant to cause a measured biological change (any one of interest). If the change occurs at low concentrations or doses, the compound is said to have high potency. If high concentrations or doses are required, the compound is said to have low potency.

• **Intrinsic Activity (Efficacy):** The maximal response caused by a compound (i.e., toxicant or drug) in any given test preparation. Intrinsic activity (efficacy) is always defined relative to a standard compound such as the endogenous ligand for a receptor or the prototypical toxicant for a specific system. For drugs, a **full agonist** causes a maximal effect equal to that of the endogenous ligand or reference compound; a **partial agonist** causes less than a maximal response. An **antagonist** binds to the same site but causes no functional response. More recently, ligands have also been classified as inverse agonists (decrease constitutive activity in the opposite direction as agonists). These issues will be discussed later in Section 19.4.

### 19.2 RECEPTOR SUPERFAMILIES

Receptors can be divided into several distinct classes based on the effector mechanisms evoked by ligand binding (Figure 19.2). Three major classes of receptors that have endogenous ligands and also bind toxicants include the G-protein-coupled receptors, the ionotropic receptors (also known as ligand-gated ion channels), and intracellular steroid receptors. In addition, the members of a superfamily of enzyme-linked receptors bind a variety of polypeptide hormones and growth factors, and they exert pleiotropic effects on cell physiology. Although these receptors are not known to bind xenobiotic toxicants, their central role in controlling cell proliferation and cell death makes it likely that they participate ultimately in the perturbations and adaptations induced by exposure to a variety of toxicants. Finally, voltage-gated ion channels are macromolecules that span the cell membrane and provide a pathway for particular ions. Although no endogenous ligands have been identified, a variety of xenobiotic toxicants act on these sites. Recent progress in the molecular cloning of receptors has demonstrated considerable amino acid sequence homology among receptors that share a common effector mode; such observations provide an additional rational basis for classification, but more importantly, they enable a search for characteristics (i.e., structural motifs) that must ultimately confer specificity of receptor-effector coupling.

#### 19.2.1 G-Protein-Coupled Receptors (GPCRs)

After binding of ligands, many neurotransmitter receptors (e.g., the dopamine, adrenergic, GABA<sub>B</sub>, metabotropic glutamate, and muscarinic cholinergic receptors)
use large guanine nucleotide-binding proteins (G proteins) as their transduction systems. Thus, binding of the ligand to the receptor ultimately changes the rate of synthesis or degradation of various cytoplasmic effectors that are modulated by these G proteins (see 1 in Figure 19.2). The G-protein-coupled receptors contain a single polypeptide chain with seven transmembrane spanning regions. There is an extracellular/intramembrane domain for ligand recognition, and cytoplasmic domains involved in coupling to G proteins. Interaction of the ligand with the receptor initiates a cascade of secondary events that are initiated by G-protein activation. This, in turn, results in the activation or inhibition of specific enzymes (e.g., adenylate cyclase or phospholipase C), with subsequent changes in the turnover of intracellular “second messengers” such as cAMP, diacylglycerol, phosphoinositols, the direct regulation of ion channels, and so on. Together, these biochemical events determine the ultimate cellular consequences of ligand binding.

The best-characterized model for receptor–G-protein interaction is derived from studies of receptors linked to the stimulation of adenylate cyclase (e.g., β2-adrenergic receptors). In the absence of ligand, the receptor is presumed to exist in a high-affinity state stabilized by the heterotrimer G protein that has GDP tightly bound to the α-subunit of the G-protein. Agonist binding to the receptor induces a conformational change in the complex of receptor and G protein, promoting the exchange of GDP for GTP. This results in the subsequent dissociation of the

![Figure 19.2. Schematic representation of some receptor superfamilies. This figure represents the common mechanisms of the four major receptor families discussed in Section 19.2. Example 1 is of the G-protein-coupled receptor. Example 2 represents the ionotropic receptor (ligand-gated ion channel) superfamily, on which multiple binding sites are known to exist (see Figure 19.1). Example 3 represents the steroid receptor superfamily. Example 4 depicts the enzyme-linked receptor superfamily.](image-url)
G-protein subunits into $\alpha$ and $\beta\gamma$ subunits. Both the $\alpha$ and $\beta\gamma$ subunits may elicit secondary events such as the activation or inhibition of the catalytic unit of adenylate cyclase.

Adenylate cyclase is considered as a second messenger that catalyzes the formation of cAMP (cyclic adenosine monophosphate) from ATP; this results in alterations in intracellular cAMP levels that change the activity of certain enzymes—that is, enzymes that ultimately mediate many of the changes caused by the neurotransmitter. For example, there are protein kinases in the brain whose activity is dependent upon these cyclic nucleotides; the presence or absence of cAMP alters the rate at which these kinases phosphorylate other proteins (using ATP as substrate). The phosphorylated products of these protein kinases are enzymes whose activity to effect certain reactions is thereby altered. One example of a reaction that is altered is the transport of cations (e.g., Na$^+$, K$^+$) by the enzyme adenosine triphosphatase (ATPase).

These processes are all possible loci for biochemical attack by toxicants. For instance, compounds like ephedrine or mescaline are alkaloids that act as ligands at GPCRs. Many drugs have been made to act at these sites. Another possible site of attack is at the G-protein level. Diphtheria and cholera toxin act on guanine nucleotide-binding proteins linked to the stimulation ($G_s$) or inhibition ($G_i$) of adenylate cyclase. Both toxins act by enzymatically transferring an ADP-ribosyl group to a G-protein subunit. In the case of $G_s$, the result is an enduring activation of adenylate cyclase, while in the case of $G_i$, ribosylation disrupts hormonal inhibition of adenylate cyclase.

19.2.2 Ionotropic Receptors (Ligand-Gated Ion Channels)

A number of receptors are transmembrane proteins whose structure incorporates an ion channel through the cell membrane (see 2 in Figure 19.2). Ligand binding presumably induces conformational changes in the receptor protein that open the channel and allow ions of particular size and charge to pass through, thus altering the ionic concentrations (Na$^+$, K$^+$, Cl$^-$, and/or Ca$^{2+}$) across the membrane. In neuronal cells, the resultant shifts in membrane potential are either depolarizing or hyperpolarizing, making the cell more or less excitable, respectively. Two prototypical examples of ionotropic receptors are the nicotinic acetylcholine receptor and the GABA$_A$ receptor (see Figure 19.1 for the latter). In both cases, molecular cloning indicates that these receptors are formed from multiple subunits that are transcribed separately and then assembled after translation. For example, the GABA$_A$ receptor, one of the two major subclasses of receptors for the neurotransmitter GABA ($\gamma$-aminobutyric acid), is known to be a polymer with four or five subunits which together form a chloride channel. Besides the loci to which GABA binds, there are several other binding sites that have been characterized and are of interest to toxicologists (see Figure 19.1).

Several receptors that recognize the excitatory amino acid glutamate (e.g., the NMDA, kainate, and quisqualate classes) represent another group of ligand-gated ion channels. The NMDA receptor has been the focus of intense interest in recent years. This receptor appears to be activated primarily by membrane depolarizations that occur during periods of heightened neuronal activity. The activated NMDA receptor channel allows both Na$^+$ and Ca$^{2+}$ to flow into the cell. The detrimental
effects of massive Ca$^{2+}$ influx have been implicated in the selective cytotoxicity that occurs in brain regions rich in NMDA receptor (e.g., hippocampus) during episodes of disinhibited neuronal activity, such as during seizures. Some years ago, a major poisoning episode in Canada involved domoic acid, a marine toxin that has sometimes contaminated commercial seafood. Domoic acid works as an ionotropic glutamate receptor ligand, apparently causing toxicity by permitting calcium influx through some members of these receptors.

### 19.2.3 Intracellular Steroid Receptors

In contrast to the preceding three categories of cell surface receptors, binding sites for diffusible steroids can be found intracellularly, although the exact anatomical locus (cytoplasm versus nucleus) remains controversial (see 3 in Figure 19.2). Cloning of several of these intracellular receptors has verified the presence of distinct steroid-binding and DNA-binding subunits. Endogenous steroid ligands include the estrogens, androgens, progestins, glucocorticoids, mineralocorticoids, and vitamin D, as well as some active metabolites of these compounds. The proximal effect of ligand occupation of steroid receptors is the activation (or disinhibition) of a DNA-binding domain, enabling the interaction of this domain with regulatory DNA sequences (promoter regions) that influence the rate of transcription of particular genes. Compared to the previously described receptor-mediated events, the changes in gene expression that are mediated by steroid binding are both slower and more enduring. One fascinating line of recent research has examined the role of glucocorticoids in promoting cellular damage during episodes of heightened neural excitability (e.g., during ischemia).

### 19.2.4 Enzyme-Linked Receptors

A large and heterogeneous superfamily of cell surface receptors is defined by the presence of a single transmembrane segment and a direct or indirect association with enzyme activity (see 4 in Figure 19.2). Ligands for these receptors include cytokines, interferons, and many growth factors. Some members of this receptor superfamily possess intrinsic catalytic activity within their cytoplasmic domain (e.g., tyrosine kinase, serine-threonine kinase, guanylate cyclase, and phosphotyrosine phosphatase). In other cases, receptors are devoid of intrinsic enzyme activity but associate directly with cytoplasmic enzymes. The intracellular “signal” generated by ligand binding of enzyme-linked receptors is conveyed by phosphorylation/dephosphorylation of various intracellular targets, or by generation of cGMP.

One important subclass of enzyme-linked receptors whose signaling pathway has been delineated recently is receptor tyrosine kinases (RTK). Two of the prototypical RTKs bind insulin and epidermal growth factor. RTK receptor structure is defined by three elements: an extracellular domain with a ligand-binding site, a single hydrophobic alpha helix that spans the membrane, and an intracellular domain with intrinsic protein tyrosine kinase activity. In most cases, RTK receptors exist as monomers in the ligand-unbound state. The binding of soluble or membrane-bound peptide or protein ligands promotes the formation of dimers. This ligand-triggered dimerization elicits autophosphorylation of specific intracellular tyrosine residues. These phosphotyrosines serve as binding sites for distinct cytosolic proteins that
contain a polypeptide domain called the Src homology 2 (SH2) domain. Slight differences in the amino acid sequence among SH2-containing proteins confer receptor specificity by enabling recognition of distinct amino acid sequences surrounding a phosphotyrosine residue in the receptor. SH2-containing proteins include a number of cytosolic enzymes and specific adapter proteins that link the RTK receptor to other signaling molecules. Ras proteins have been shown to be involved in the signal transduction pathway elicited by ligand binding to a number of RTKs, whose ultimate effects include regulation of cell growth, differentiation, and metabolism. The demonstration of RTK and Ras involvement in human cancers has sparked great interest in further elucidation of this important signaling cascade.

19.2.5 Additional Cell Surface Targets for Toxicants

Electrical potentials in excitable cells arise from the differential localization of a number of ions (e.g., Na⁺, K⁺, Ca²⁺, and Cl⁻) across the semipermeable plasma membrane. Selective changes in the permeability of the membrane to certain ions produce alterations in the membrane potential that ultimately are responsible for the propagation of electrical signals between cells. Voltage-gated ion channels are macromolecules that span the cell membrane and provide a pathway for particular ions (similar to 2 in Figure 19.2). The mechanism for voltage dependency of these channels currently is unclear, although it probably involves a conformational change induced by altered interactions among charged amino acid residues of the channel protein. The best-characterized voltage-gated conductance channel is the Na⁺ channel. It is a heavily glycosylated protein formed of a membrane spanning α-subunit and one or two polypeptide β-units that are situated on the extracellular membrane face. There are a number of binding sites present on voltage-gated channels that alter their function. Although no endogenous ligands for these sites have been identified, a variety of xenobiotic toxicants act on these sites. The puffer fish toxin tetrodotoxin and the paralytic shellfish toxin saxitoxin bind to the same site near the extracellular opening of the Na⁺ channel, thereby blocking Na⁺ transport through the channel. Several lipid-soluble anesthetics (e.g., lidocaine) appear to bind to a hydrophobic site on the protein. Other toxicants act to increase spontaneous channel opening (e.g., aconitine) or channel closing (e.g., scorpion toxins).

19.2.6 The Importance of Understanding Toxicant–Receptor Interactions

The approaches outlined in this chapter are important for several distinct reasons. First, they can be applied to answer mechanistic questions about the proximal actions of a toxicant. The methods and strategies of such approaches, along with appropriate examples, will be provided later in this chapter. A second distinct rationale also is of importance. Toxic insult (from nervous system damage or chronic drug administration) may cause compensatory changes that include alterations in receptor characteristics. Such changes may be essential to understanding the toxicant-induced physiological changes and, possibly, essential to the chain of events initiated by the toxicant. Finally, finding specific receptors for toxicants often provides important information that can be useful in understanding not only the toxicant, but also cellular function. Consider that finding a receptor for morphine certainly provided information about its mode of action; but more importantly, it
opened a new horizon in understanding how ligands acting at that family of receptors affected nervous system function.

It will become apparent in the material that follows that much of the present discussion bears a great similarity to elementary enzyme kinetics. In fact, one can often understand a great deal about relevant toxicological phenomena by applying simple mass action considerations. The emphasis in this chapter shall be on the recognition of a high-affinity toxicant with a macromolecule, under the assumption that one is dealing with a simple reversible phenomenon. In fact, this is often not the case. With enzyme substrates, the reaction is often not just bimolecular, and a variety of mechanisms can be invoked to yield product. Similarly, in many cases where the reaction is between a neurotransmitter receptor and a ligand, various phenomena such as internalization or trapping can complicate this picture.

19.3 THE STUDY OF RECEPTOR–TOXICANT INTERACTIONS

19.3.1 Development of Radioligand Binding Assays

As noted above, many neurotoxicants bind specifically to sites on neurons or glia; consequently, receptor binding assays have become a useful method to study toxicants that have high-affinity interactions with a binding site. Prior to the 1970s, the study of receptors was limited largely to indirect inferences from measurement of physiological responses suspected of being mediated by the receptor of interest. The availability of radiolabeled drugs with high affinity for specific receptors enabled the development of binding assays to assess receptor–ligand interactions in isolated tissue preparations. In the two decades since these methods were introduced, in vitro binding assays have become routine. Advances in molecular biology have paved the way for new generations of techniques toward this same end, yet the radioreceptor methods described below are still used widely and remain a powerful tool when one wishes to elucidate the mechanism of action of small molecules.

The essential ingredient in this technique is the availability of radiolabeled ligand; this radioligand is a molecule in which one or more radioactive atoms has been incorporated into the structure without significantly changing its receptor recognition characteristics. Often, scientists can choose among several radiolabeled agents specific for a particular receptor type. The choice of the type of radioactive isotope is governed by the half-life of the isotope, the amount of receptor in a tissue preparation, and the affinity of the receptor for the radioligand. In practice, specific activities of at least 10 Ci/mmol are required, meaning that $^3$H, $^{125}$I, $^{35}$S, and $^{32}$P can be used, whereas $^{14}$C cannot. As an example, a tritiated ligand has a much lower molar specific activities (~30 Ci/mmol) compared to a similar ligand with a single $^{125}$I atom (~2200 Ci/mmol); the former thus affords lower sensitivity. On the other hand, the replacement of hydrogen with tritium is unlikely to alter receptor recognition characteristics, whereas addition of a bulky iodine atom may have profound effects. Nonetheless, whereas ligands using $^3$H are used commonly, $^{125}$I, $^{35}$S, and $^{32}$P containing molecules also are sometimes employed.

Radioligand binding assays are performed typically in solutions of extensively washed membranes prepared from tissue homogenates or cultured cells. Assays of receptor binding to slide-mounted thin tissue sections (quantitative receptor
autoradiography) also have become popular, and they are useful particularly for the study of the anatomical localization of binding sites. In both cases, tissue is incubated with radioligand for a specific duration, followed by measurement of the amount of ligand–receptor complex. Accurate assessment of the amount of radioactivity bound to receptors requires a method to separate bound ligand from unbound and a nonbiased estimate of the amount of ligand bound specifically to the receptor of interest.

There are three criteria that are important to determine if a suspected toxicant binding site is biologically meaningful, and also whether it may be a receptor with a known physiological function. (These issues are discussed in greater detail later in the chapter.) There should be a finite number of receptors in a given amount of tissue; thus, the binding should be saturable. As increasing amounts of the radioligand are added to the tissue, the amount of specific binding should plateau [see Figure 19.6 (top) and discussion in Section 19.3.2c]. If the binding site is a known or suspected physiological receptor, other drugs or toxicants that bind to the same receptor should compete with the radioligand for receptor occupancy. The selectivity and potency of this binding should parallel the way they enhance or block the physiological effects of the toxicant believed to be mediated through the receptor. This is usually ascertained by use of competition assays (described in detail in Section 19.3.4). Finally, the kinetics of binding often will be consistent with the time course of the biological effect elicited by the toxicant.

19.3.2 Equilibrium Determination of Affinity (Equilibrium Constants)

19.3.2a Law of Mass Action and Fractional Occupancy. Although toxicant–receptor interactions may involve very complex models, they often follow an elementary mass-action model that results in algebraic expressions similar to those seen in Michaelis–Menten enzyme kinetics. Essentially, this model (Figure 19.3) is the reversible interaction of a toxicant with a binding site (i.e., “receptor”). Although the interaction may generate physiological responses, for receptor analyses it can be modeled without making some of the assumptions needed in enzyme kinetics (e.g., initial velocity conditions). Thus, we can illustrate this model as shown in Figure 19.3.

Thus, for a toxicant (ligand) interacting with a single (or homogeneous population) of receptor(s), mass-action principles require:

\[
\text{Ligand + Receptor} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{Ligand – Receptor} \quad (19.1)
\]

For any given system, there is a tendency for ligand and receptor to remain associated (the association rate constant \(k_{\text{on}}\)) and for the ligand–receptor complex to

![Figure 19.3. Simplest model of how toxicant–receptor interactions can lead to functional consequences.](image)
dissociate (the dissociation rate constant $k_{\text{off}}$). Equilibrium is reached when the rate of formation of new ligand–receptor complexes equals the rate at which existing the ligand–receptor complexes dissociate. Thus, equilibrium is reached when the rate of complex formation equals the rate of complex dissociation, as shown algebraically in Eq. (19.2).

$$\text{(19.2)}$$

From basic principles of mass action, we know the relationship between these rate constants and the association ($K_A$) and dissociation ($K_D$) equilibrium constants:

$$\frac{1}{K_A} = \frac{k_{\text{off}}}{k_{\text{on}}} = K_D$$  \hspace{1cm} (19.3)

Thus, we can rearrange Eq. (19.2) to define the equilibrium dissociation constant $K_D$:

$$\frac{[\text{Ligand}][\text{Receptor}]}{[\text{Ligand} \cdot \text{Receptor}]} = \frac{k_{\text{off}}}{k_{\text{on}}} = K_D$$  \hspace{1cm} (19.4)

From this, it is clear that the $K_D$ is the concentration of ligand that, at equilibrium, will cause binding to half the receptors. The $K_D$ is the equilibrium dissociation constant, whereas the $k_{\text{off}}$ is the dissociation rate constant. A summary of these definitions (and their chemical units) is shown in Table 19.1.

A term that is sometimes useful for biological scientists is fractional occupancy. Based on the law of mass action, this term describes (at equilibrium) receptor occupancy as a function of ligand concentration. Specifically:

$$\text{Fractional occupancy} = \frac{[\text{Ligand} \cdot \text{Receptor}]}{[\text{Total receptor}]}$$  \hspace{1cm} (19.5)

Since $[\text{Total receptor}] = [\text{Receptor}] + [\text{Ligand} \cdot \text{Receptor}]$, then

$$\text{Fractional occupancy} = \frac{[\text{Ligand} \cdot \text{Receptor}]}{[\text{Receptor}] + [\text{Ligand} \cdot \text{Receptor}]}$$  \hspace{1cm} (19.5a)

From the equation for $K_D$ derived above, it is seen that

$$[\text{Receptor}] = \frac{K_D \cdot [\text{Ligand} \cdot \text{Receptor}]}{[\text{Ligand}]}$$  \hspace{1cm} (19.6)

### Table 19.1. Chemical Constants and Their Units

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<tr>
<th>Variable</th>
<th>Name</th>
<th>Units</th>
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<tr>
<td>$k_{\text{on}}$</td>
<td>Association rate constant or “on” rate constant</td>
<td>M⁻¹min⁻¹</td>
</tr>
<tr>
<td>$k_{\text{off}}$</td>
<td>Dissociation rate constant or “off” rate constant</td>
<td>min⁻¹</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Equilibrium dissociation constant</td>
<td>M</td>
</tr>
</tbody>
</table>

2
One can substitute this value for [Receptor] in both the numerator and the denominator of the equality for fractional occupancy and, by simplifying, obtain the following. (Try to derive this yourself on a piece of paper.)

\[
\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}]} D + K_D^{-1} \quad (19.7)
\]

Equation (19.7) assumes that the system is at equilibrium. To make sense of it, think about a few different values for [ligand]. When [ligand] = 0, the fractional occupancy equals zero. When [ligand] is very, very high (i.e., many times the $K_D$), the fractional occupancy approaches 100%. When [ligand] = $K_D$, fractional occupancy is 50% (just as the Michaelis–Menten constant $K_m$ describes the concentration of enzyme substrate that gives half-maximal velocity).

**Fractional Occupancy Is an Important Concept for Several Reasons.** For example, if one knows the $K_D$ of a ligand for a target site, one can estimate the occupancy, and hence the effects, that might be caused by a given concentration of the toxicant. Similarly, in experimental toxicology, it permits one to choose concentrations of a test compound that are likely to cause the desired effects without being unnecessarily high and causing undesired secondary effects. Finally, this equation makes clear why semilog dose response curves are so useful in toxicology. Table 19.2 illustrates the fractional occupancy (FO) values calculated for a hypothetical toxicant with a $K_D = 1$ nM (an arbitrary value chosen as an easy example). (Be sure that you can calculate the FO values as shown in this table.)

One can then plot these data as shown in Figure 19.4.

**19.3.2b The Theoretical Basis for Characterizing Receptors Using Saturation Radioligand Assays.** Radioreceptor assays were first developed in the early 1970s. They were based on two simple, but very elegant, concepts.

1. If a ligand had high affinity for a macromolecular target (as had been shown by classical pharmacological studies over many decades), it should be thermodynamically possible to measure the binding of the ligand to the receptor without
the need to perform equilibrium dialysis (the only method then used) as long as one could separate the ligand–receptor complex from the free ligand.

2. By labeling ligands with appropriate radioactive atoms, one could detect the ligand–receptor sensitively and rapidly. (This was the key point, since chemical methods were neither sufficiently sensitive, nor inexpensive, for this use.)

We begin with the simple law of mass action shown in Eq. (19.1):

\[
\text{Ligand + Receptor } \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{Ligand – Receptor}
\]  

(19.1)

We developed the following equation:

\[
\frac{[\text{Ligand}][\text{Receptor}]}{[\text{Ligand} \cdot \text{Receptor}]} = \frac{k_{\text{off}}}{k_{\text{on}}} = K_D
\]  

(19.4)

It is useful to replace some of these terms with equivalent ones that are common lab jargon in the field and that reflect the measurements that are actually made in characterizing receptors. These names are as follows:

The \([\text{Ligand–Receptor}]\) complex is called “Bound” or “B”.

The unbound ligand is called “Free” or “F”.

As noted, both \(B\) and \(F\) are measurable experimentally. We can then substitute in the above equation [originally Eq. (19.4)] with these terms and obtain the following:

\[
\frac{F \cdot [\text{Receptor}]}{B} = K_D
\]  

(19.8)

Since \(F\) and \(B\) are independent variables, and we wish to solve for \(K_D\), it is necessary to be able to quantify the unbound receptor. Yet this is almost always technically impossible or impractical. On the other hand we know the following:

\[
[\text{Total receptor}] = [\text{Receptor}] + [\text{Ligand–Receptor}]
\]  

(19.9)

**Figure 19.4.** Plot of fractional occupancy (FO) versus the [ligand] and the log [ligand]. This provides the theoretical basis for the utility of semilog plots; a sigmoidal curve results that allows maximal visual extrapolation of information from the most biologically meaningful part of the dose–response curve.
The [Total receptor] present is commonly termed the “$B_{\text{max}}$” (i.e., the maximal number of binding sites). Thus,

$$B_{\text{max}} = [\text{Receptor}] + B$$  \hspace{1cm} (19.10)

Equation (19.10) can be rearranged:

$$[\text{Receptor}] = B_{\text{max}} - B$$  \hspace{1cm} (19.11)

We now take Eq. (19.11) and substitute the right-hand side of Eq. (19.11) for the [Receptor] in Eq. (19.8) as follows:

$$FB \cdot K = [\text{Receptor}]$$  \hspace{1cm} (19.8)

$$FB \cdot B \cdot K - = B_{\text{max}}$$  \hspace{1cm} (19.12)

We can simplify this by multiplying both sides by $B$ and expanding the left-hand parenthetical expression to yield

$$FB \cdot B \cdot K \cdot B = B_{\text{max}} \cdot B$$  \hspace{1cm} (19.13)

With simple rearrangement, we get

$$K \cdot B + F \cdot B = B \cdot B_{\text{max}}$$

This can be factored to

$$B(K_D + F) = B \cdot B_{\text{max}}$$

resulting in the following equation:

$$B = \frac{B \cdot B_{\text{max}}}{K_D + F}$$  \hspace{1cm} (19.14)

This equation should look familiar, because it is functionally identical to the Michaelis–Menten equation of enzyme kinetics. This equation also should make clear the experimental design to be used in determining $K_D$ and $B_{\text{max}}$ using saturation isotherms. We have as the independent variable $[F]$ and as dependent variable $B$. A successful experiment should permit the estimation of the two biologically meaningful constants: $K_D$ and $B_{\text{max}}$.

The $K_D$ and $B_{\text{max}}$ are of interest to toxicologists and other biological scientists because of the information they convey. The $B_{\text{max}}$ defines the number of available binding sites. This can provide clues about whether or not a particular tissue may bind the toxicant, or whether a toxicant alters the available number of binding sites (e.g., through killing cells or causing compensatory responses). The $K_D$ defines the affinity of the toxicant.
for the binding site. This provides clues about the likelihood of the toxicant working through the mechanism being tested, and also clues about whether intoxication alters the binding site through some compensatory or toxic response.

19.3.2c  Experimental Forms of Data from Saturation Radioligand Assays.
As we did with fractional occupancy, we can predict the data that would result from a toxicant-receptor interaction that was modeled by Eq.  (19.14). Let's see what theoretical data would look like if we assume (for the sake of easy calculations) that the $B_{\text{max}} = 100$ and the $K_D = 1\text{ nM}$. (This is a similar exercise to what we did with fractional occupancy.) Picking arbitrary $[F]$ concentrations, Table 19.3 shows the derived values [try this yourself using Eq. (19.14)].

If we plot these data in either a linear or semilogarithmic format, a nonlinear plot results as shown in Figure 19.5 (panel A). In the era when computers were not readily available, it was recognized that one could linearize Eq. (19.14) by several simple algebraic rearrangements. Some of these (and their names) are shown in Figure 19.5 with both their common name and how a typical plot of the linearized data would appear. If data are near-ideal (e.g., the calculated data in Table 19.3), then all of these plots will yield identical results for $K_D$ and $B_{\text{max}}$ when their slope and intercept(s) are calculated. In practice, they each are affected differently by sources of experimental variance. In any event, direct calculations should now be done using computerized nonlinear regression [e.g., based on Eq. (19.14)]. This can be done with specialized programs such as Prism (GraphPad, Inc), or with general-purpose scientific plotting software (e.g., SigmaPlot has specific radioligand binding modules).

One of the reasons, however, that Scatchard plots still are used (or at least often expected for publication) is that they permit quick visual estimates of the $B_{\text{max}}$ (i.e., the $B_{\text{max}}$ is the $X$-intercept of the extrapolated line) and of relative toxicant affinities (reflected as the negative reciprocal of the slope). The steeper the slope, the smaller the $K_D$, and the greater the affinity of the toxicant for the receptor.

Figure 19.6 (bottom) shows the Scatchard transformation of the saturation data shown in Figure 19.6 (top). In this case, a linear regression analysis adequately fits these data. As noted above, the slope of the regressed line is the negative reciprocal of the $K_D$, and the $x$-intercept is the $B_{\text{max}}$. Curvilinear Scatchard plots can indicate the presence of multiple populations of receptors with differing affinities for the

| TABLE 19.3. Derived Values for Theoretical Saturation Plot |
|-----------------|-----------------|-----------------|
| $F$ (nM) | $\log [F]$ | $B$ |
| 0.01 | -2 | 0.99 |
| 0.03 | -1.52 | 2.91 |
| 0.1 | -1 | 9.09 |
| 0.3 | -0.52 | 23.08 |
| 1 | 0 | 50.00 |
| 3 | 0.48 | 75.00 |
| 10 | 1 | 90.91 |
| 30 | 1.48 | 96.77 |
| 100 | 2 | 99.01 |
Figure 19.5. Some linear transformations of Eq. (19.14) and the resulting plots.

toxicant. Alternatively, they may indicate positive or negative cooperativity (producing upwardly or downwardly "concave" plots), where the binding of one toxicant molecule influences the binding of subsequent molecules.

A potential problem associated with Scatchard analysis occurs when the range of radiolabeled toxicant concentrations used is too narrow, such that the highest concentration does not equal or exceed the $K_D$ (that concentration of toxicant which results in the occupation of one half of the total binding sites). Although not apparent from a Scatchard plot of such data, estimates of $B_{max}$ under such conditions are prone to significant error.

Two other possible transformations of saturation binding data mentioned earlier are the Hofstee ($B$ versus $B/F$) and Woolf Plots ($F/B$ versus $F$). As with the Scatchard equation, these equations can be derived from algebraic manipulations of the equations listed above.
19.3.3 Kinetic Determinations of Equilibrium Constants

Although equilibrium methods are used most commonly to estimate parameters of receptor binding, separate kinetic analyses should be employed during the initial characterization of toxicant binding—for example, when a novel toxicant radioligand becomes available or an established radioligand is planned for use in a new tissue type or species. Several of the general references cited at the end of this chapter provide an excellent detailed discussion of kinetic binding methods and the derivation of the associated equations. Briefly, kinetic experiments to determine

Figure 19.6. Characterization of a receptor using a saturation isotherm with a radioligand. (Top) In this experiment, increasing amounts of radioligand are added to tubes containing a constant amount of tissue. A duplicate series of tubes also includes an excess (>100-fold) of a known competitor for the receptor being studied. The solid lines indicate the total amount of radioligand bound to the receptor (Total Bound) and the nonspecific binding (Nonspecific). The nonspecific binding is due to physicochemical interactions of the radioligand with proteins or lipid (e.g., dissolving into membrane lipids). The specific binding (the term of interest) is obtained by subtracting the Nonspecific from the Total Bound. As predicted by Eq. (19.14), a plot of specific binding versus ligand added yields a rectangular hyperbola. (Bottom) A Scatchard plot of these data (based on equation shown in Figure 19.5, panel B). With these data, the extrapolated density of receptors is approximately 16 fmol. The KD could be calculated based on −1/slope.
association and dissociation rate constants are conducted separately. Association rate is estimated by measuring the amount of specific toxicant bound as a function of time; assuming that the amount of free radioligand does not change appreciably over time, then a pseudo-first-order rate equation is used to estimate $k_1$. The rate of dissociation of the toxicant is determined by incubating tissue with toxicant, allowing the reaction to proceed to equilibrium, and then stopping it either by infinite dilution or by the addition of an excess of nonradiolabeled ligand. This stops the forward association reaction, so that measurement of the amount that remains bound as a function of time can be attributed solely to the dissociation between receptor and toxicant. The rate of change of the receptor–ligand complex over time is used to estimate $k_{-1}$, the dissociation rate constant.

In theory, $K_D$ (i.e., $k_{-1}/k_1$) should be the same whether determined by kinetic or equilibrium approaches. In practice, however, moderate differences arise that are often attributed to technical problems associated with separating bound from free rapidly without losing a significant proportion of receptor–toxicant complex. This problem is troublesome, particularly when estimating the amount bound at early time points in association or dissociation experiments, when the amount of bound ligand is changing rapidly. Large differences between the $K_D$ as determined in saturation and kinetic experiments, however, may indicate that the reaction is more complex than a simple bimolecular reversible reaction.

### 19.3.4 Competition Binding Assays

Competition binding assays, also referred to as indirect binding studies, involve measuring the competition between a radioligand and an unlabeled drug for a specific receptor site. The concentration of the radioligand is held constant, while the concentration of the unlabeled drug is varied over a wide range of concentrations. If both drugs compete for the same receptor site, then the amount of radioligand bound will decrease systematically as the concentration of the unlabeled drug increases (i.e., as more of the unlabeled drug binds). Competition studies are particularly attractive because they allow the study of many drugs that are not suitable for radiolabeling and direct binding measurements due to their low affinity. Applications of this technique include determining the binding specificity of a radiolabeled toxicant, comparing the potencies of a series of unlabeled toxicants for the radiolabeled receptor site, and providing a reliable estimate of nonspecific binding.

Data from competition binding experiments are plotted routinely as the percent inhibition of specific radioligand binding (total – nonspecific) as a function of log$_{10}$ of the concentration of competitor. The 100% binding value is defined as the amount of specific radioligand binding in the absence of competitor. When comparing the potency of several compounds, it is useful to plot several competition ("displacement") curves together because the relative position of the competition curves for various ligands will be related to their potency in displacing the radioligand, with the leftmost curves representing the most potent competitors.

Figure 19.7 (top) illustrates competition binding data of two compounds that can compete for radioligands that label the receptor for the neurotransmitter dopamine (specifically, the D$_1$ receptor subclass). It can be seen that Compound A can eliminate specific binding at concentrations about 100-fold lower than required for Compound B. In toxicological (and pharmacological) terms, one would say that
Compound A is more potent than Compound B in this assay (or more rigorously, A has higher affinity). Specifically, higher concentrations of Compound B are required to cause 50% inhibition of binding than are required of Compound B. One other point should be obvious in these data in Figure 19.7. The slopes in the top curves are clearly not parallel for Compound A and Compound B, with the latter being more shallow. The shape of the competition curves also provides important information concerning the nature of the interaction between receptor and ligand, as will be described in Sections 19.3.3.5 and 19.3.6.

The relevant kinetic model for competition experiments with a radiolabeled drug [D] and an unlabeled competitor [I] is shown in the two equations in (19.15). When both sets of reactions have proceeded to equilibrium, the net rate of formation of both (DR) and (IR) are zero, and the following Eq. (19.16) can be derived from mass-action principles.
TOXICANT–RECEPTOR INTERACTIONS: FUNDAMENTAL PRINCIPLES

\[
\begin{align*}
D + R & \rightleftharpoons_{k_{on}}^{k_{off}} D \cdot R \\
I + R & \rightleftharpoons_{k_{on}}^{k_{off}} I \cdot R
\end{align*}
\] (19.15)

\[
I \cdot R = \frac{B_{\text{max}} \cdot I}{I + K_I \cdot \left(1 + \frac{D}{K_D}\right)}
\] (19.16)

In Eq. (19.16), \(K_I\) is the equilibrium dissociation constant of the unlabeled competitor, while \(K_D\) is the dissociation constant of the radioligand. \(K_I\) values of different toxicants can be compared to determine the rank order of potency of toxicants in competing for the radiolabeled receptor site, where a low \(K_I\) indicates a high potency. \(K_I\) values are routinely determined from experimentally derived IC50 values (the concentration of inhibitor that produces a 50% reduction in total specific binding of the radiolabeled drug). Although IC50 values may be estimated (with considerable error) from visual inspection of plots such as in Figure 19.7, more rigorous techniques are applied typically, including iterative nonlinear regression analysis and indirect Hill plots (see Section 19.3.4b).

19.3.4a The Cheng–Prusoff Equation. For purely competitive interactions (that fit the model shown in Figure 19.1), the relation between the IC50 and the \(K_I\) is described by the Cheng–Prusoff equation:

\[
K_I = \frac{IC50}{\left(\frac{L}{K_D}\right) + 1}
\] (19.17)

where \(L\) is the concentration of the radioligand having affinity \(K_D\). If the concentration of the radioligand is equal to its \(K_D\), then the denominator becomes 2 and the \(K_I\) of the competitor is equal to one-half of the IC50. In contrast, if the concentration of radioligand is extremely low, then the denominator becomes unity, and the \(K_I \Rightarrow IC50\).

It is important to note that the application of the Cheng–Prusoff equation requires several assumptions: (1) Both radioactive and competitor ligands are interacting reversibly with a single population of sites (bimolecular reactions); (2) the reaction is at steady state; (3) nonspecific binding is accurately estimated; and (4) the concentration of receptor is much less than the \(K_D\) for the drug or the competitor. It is important to recognize that there are really many Cheng and Prusoff equations as described in their oft-cited paper 1973 paper. The one commonly cited is only applicable to competitive bimolecular reactions following the model shown in Eq. (19.1).

19.3.4b The Hill Plot. The Hill plot was used frequently to determine whether obtained binding data deviate significantly from what would be expected from a simple reversible bimolecular reaction. The Hill transformation was developed originally to describe the cooperativity exhibited when \(O_2\) bound to hemoglobin. Hill plots can be direct or indirect, depending on whether the data are obtained from saturation or competition experiments, respectively. The Hill equation can be derived from elementary enzyme kinetic principles.
The more generalized form of the Michaelis-Menten equation is shown in Eq. (19.18):

\[ B = \frac{B_{\text{max}} \cdot [D]^n}{K_D + [D]^n} \]  

(19.18)

where \( n \) reflects whether or not the binding sites behave as a single homogeneous population rather than multiple populations or interactive sites.

If we do a series of algebraic transformations, we can arrive at the commonly used Hill equation:

\[ \log \left( \frac{B}{B_{\text{max}} - B} \right) = n \cdot \log[D] - \log K_D \]  

(19.19)

As can be seen by inspection, the Hill equation (19.19) can be plotted as a straight line in the form \( y = mx + b \), where \( y \) is the “pseudo-logit” term on the left-hand side of Eq. (19.19), and \( x = \log[D] \). In such a plot, the slope is equal to \( n \) (often called \( n_H \); the Hill number) and the \( y \) intercept is equal to \( -\log K_D \). In the case of saturation binding data, \( K_D' \) is the apparent equilibrium dissociation constant whose value reflects both the intrinsic \( K_D \) and the factors that determine how the binding of one ligand molecule affects the binding of subsequent molecules. If such sequential binding effects are not present, the \( n_H = 1 \), and \( K_D' \) will equal the \( K_D \). Alternatively, when \( n_H \) is significantly different from unity, then \( K_D' \) is not equivalent to \( K_D \). An equation of similar form (but opposite slope) can be derived for competition experiments (see below).

19.3.4c Visual Inspection of Binding Data. There are several important inferences that scientists make from examination of Hill plots. If the interaction of a ligand and its receptor follows the simple bimolecular reaction shown in Eq. (19.1), the value of the Hill coefficient \( n \) (or more commonly often \( n_H \)) will equal 1. (Please note, however, that it is usually impossible experimentally to distinguish between a single population of receptors versus several receptors having nearly similar or identical \( K_D \)'s for the ligand.) A Hill coefficient significantly less than 1 suggests a more complex situation. These possibilities include interaction of the ligand with multiple populations of binding sites (e.g., different receptors), a single class of receptor that exists in different affinity states, or even negative cooperativity among the same receptors. Alternatively, a Hill coefficient significantly greater than 1 might be obtained when the binding sites exhibit positive cooperativity.

In toxicology, an even more common use of Hill plots is for competition assays. In contrast to the direct Hill plot [from Eq. (19.19)], competition assays use indirect Hill plots. These have essentially the same form and interpretation, but have negative rather than positive slope. Again, an indirect Hill coefficient \( (n_H) \) of \(-1\) is consistent with (although it does not prove) the notion that both competitor and radioligand are interacting with one population of receptors via a simple reversible bimolecular reaction. Similarly, an \( n_H \) significantly less in magnitude than \(-1\) (e.g., \(-0.6\)) is consistent with multiple receptors or negatively cooperative receptors.

Figure 19.7 (bottom) shows the Hill plots from two of the competition curves illustrated in Figure 19.7 (top). In one case, \( n_H = -1 \), whereas in the second case it
is significantly lower (approximately −0.6). The most parsimonious explanation for these data is that the binding of Compound A is occurring to a homogeneous population of binding sites, whereas that of Compound B is to multiple or negatively cooperative binding sites.

Practicing scientists are able to make reliable estimations by visual inspecting competition curves and looking at the steepness of those curves. Such a visual inspection is based on the following line of reasoning between the apparent steepness of a competition curve and the derived Hill coefficient. First, consider that the “indirect” Hill equation can take the following form:

\[
\log \left( \frac{B}{100 - B} \right) = n \cdot \log[D] - n \cdot \log IC50
\]

(19.20)

where 100 represents the total binding (i.e., 100%) of the radioligand in the absence of competitor and \( B \) represents the percent of total binding found at each concentration of competitor \([D]\). For our purposes, we shall ignore the intercept term in this equation.

From this equation, we can construct an example that is extremely useful in visually examining receptor data, indeed any data fitting the simple mass action model of Eq. (19.1). This means that a toxicant under study is competing with the radioligand for one, and only one, population of sites. By definition, the Hill slope (often called \( n_H \)) must equal 1. It turns out that by memorizing the numbers “9” and “91,” one can do a very useful preliminary analysis of data fit to such a mass-action model. The reason for this is as follows:

At 91% of total binding, the value for the left-hand \( \log \left( \frac{B}{100 - B} \right) \) part of Eq. (19.20) can be calculated as follows:

\[
\log \left( \frac{91}{100 - 91} \right) = \log \left( \frac{91}{9} \right) = \log(10.111) = 1.005
\]

One can perform the same calculations for 9% of total binding:

\[
\log \left( \frac{91}{100 - 9} \right) = \log \left( \frac{91}{9} \right) = \log(0.989) = -1.005
\]

This means that the \( \Delta \) (change) in the left-hand side of the equation when going from 91% to 9% of total binding will be 1.005 − (−1.005) = 2.01 Hill units. Remember, as shown in Eq. (19.20), the Hill number \( n_H \) is a slope function, in which the denominator is the log of the concentration range. If \( n_H = 1 \) [that is, a system that meets the constraints of the model of Eq. (19.1)], then the log of the concentration range to cause this change from 91% to 9% total binding must also have a value of 2.01. Of course, the antilog of 2.01 is -100. Thus, if a curve has “normal” steepness [i.e., it fits Eq. (19.1)], then a change from 91% to 9% of binding must occur with a change in concentration of 100-fold. This means that one can visually inspect dose-response data to estimate whether the 91% to 9% range spans a two log order (i.e.,
100-fold) concentration range. This is a very useful way to estimate whether data are consistent with the bimolecular model.

If the change from 9% to 91% requires more than a 100-fold concentration change, then the curve is "shallow" and cannot be explained by the model in Eq. (19.1). If the change from 9% to 91% requires less than a 100-fold concentration change, then the curve is "steep" and also does not meet the model of Eq. (19.1). Of these two situations, the former is more common. One common example of shallow competition curves involves the binding of ligands to G-protein-coupled receptors (GPCRs; see 0). In such cases, the shallow steepness of the curve often is attributed to the existence of a single receptor population that exists in two interconvertible affinity states. According to this model, GPCRs exist in a complex with G proteins and have GDP bound to the $\alpha$-subunit of the G protein. These receptors are in a high-affinity state for agonists. Some of the receptors are not associated with G proteins, and they exist in a low-affinity state for agonists. Thus, normally an agonist competition curve looks shallow because it detects two populations of binding sites having very different affinity.

With these GPCRs, the addition of GTP (or related guanine nucleotide analogs) to an assay system can convert those receptors in a high-affinity to a low-affinity state by displacing the GDP. Thus, one goes from having two sites (one with high and one with low affinity for agonists) to one site (all low affinity). Experimentally, the competition curve changes from a shallow curve (due to two different populations of binding sites) to a curve of normal steepness (representing only the low-affinity site). Moreover, the curve shifts rightward. Thus, one can, even by visual inspection, test hypotheses about the underlying model. Since dose–response relationships are at the heart of toxicology, it is important to be able to make such estimations facilely.

Finally, two practical points relative to Hill plots also should be made. As can be seen in Eq. (19.20), at the IC50 of a competitor, the left-hand term will have a value of 0. Hill plots are used frequently to calculate the IC50 graphically; this value can then be substituted into the Cheng–Prusoff equation to determine a $K_I$ value (see Section 19.3.3.4). Experimentally, those data representing less than 5%, or more than 95%, radioligand occupancy are of little use since at these extremes, systematic or random sources of experimental error (e.g., counting error, pipeting error) make use of these points problematic.

19.3.4d Complex Binding Phenomena. In many cases, the assumption that a toxicant is interacting with a single population of binding sites is not tenable, as indicated by curvilinear Scatchard plots or Hill coefficients different from unity. A lengthy discussion of the alternative mathematical models used to describe possible receptor–toxicant interactions in such cases is beyond the scope of this chapter. Briefly, such analyses rely on powerful statistical computer programs (i.e., Prism or LIGAND) that can determine whether binding data can be modeled with greater precision by assuming the existence of two (or more) receptor sites, rather than one. If so, separate parameter estimates can be obtained for the two sites. Although two-site analysis is used frequently, the ability to resolve more than two receptor sites currently is limited. In all cases, it is important to emphasize that such analyses should be used to generate testable hypotheses about the multiple sites, rather than as proof for their existence. Finally, it should be recognized that a number of
technical problems (failure to reach equilibrium at low drug concentrations) could produce data consistent with multiple sites; thus, a careful consideration of potential artifacts is wise prior to entertaining the idea of multiple receptor populations.

19.3.5 How to Conduct a Radioreceptor Assay

19.3.5a Methods to Separate Bound from Free Radioligand. Three methods have been used traditionally to separate bound from free ligand in radioreceptor assays. Dialysis, originally widely used to study enzyme–substrate interactions, has several technical problems that prevent its wider use. These include degradation or sticking of receptor or ligand, the cumbersome nature of assays when large numbers of samples are needed, and the long time to obtain equilibrium. Centrifugation to separate ligand bound to tissue receptors from free ligand in solution is limited because the ligand dissociates from the receptor during pelleting and a large amount of drug receptor complex is therefore lost. There is an inverse relationship between the affinity of the ligand for the receptor (i.e., \(1/K_D\)) and the time allowable for centrifugation. One can calculate this time based on the \(K_D\) of the radioligand and the derived rate constants; the relationship will be logarithmic. For example, assuming that separation must be complete in \(0.15t_{1/2}\) to avoid losing more than 10% of ligand–receptor complex, then centrifugation must be accomplished in 10 sec for a ligand with a \(K_D\) of 10 nM and in 1000 sec for a ligand with a \(K_D\) of 0.1 nM.

The most widely used method to separate bound from free radioactivity is vacuum filtration. The suspension of tissue homogenate is diluted and is rapidly aspirated through filters that retain the tissue membranes but not the solvent. With a sufficiently high ligand affinity for the receptor, several washes of the filter can be used to achieve low levels of nonspecific binding. The use of ice-cold buffer for these washes decreases the dissociation rate of the receptor–ligand complex, permitting more extensive washing with lower nonspecific binding. The ability to prepare large numbers of samples simultaneously through the use of commercially available filtration systems is a major advantage of this method. An excellent summary of the factors that should be considered in choosing a separation method is provided by Limbird (2004).

19.3.5b Methods to Estimate Nonspecifically Bound Radioligand. It is important to recognize that the total amount of radioligand bound to tissue represents ligand not only bound to high-affinity receptors, but also bound “nonspecifically” to other tissue components as well as to assay materials (filter paper, glass or plastic tubes, etc.). In contrast to receptor-bound ligand, nonspecifically bound ligand is assumed to be nonsaturable; nonspecific binding continues to increase as radioligand concentration increases. Most nonspecific binding often is believed to be due to hydrophobic interactions between the radioligand and lipophilic tissue constituents, such as membrane phospholipids. One may conceptualize this process as a sort of “dissolving” of the radioligand into biological membranes. Thus, while this process will be slow (“low affinity”), in an aqueous environment it will be both essentially irreversible and of high capacity relative to the limited number of specific receptors.

A method used commonly to estimate the nonspecific binding component involves measuring the amount of radioligand bound in the presence of a large
excess of unlabeled ligand (>100 \* K_D). This large excess should compete for receptor sites, preventing almost all binding of radiolabeled ligand. Conversely, the model discussed in the previous paragraph would predict that radioligand bound to nonspecific components should be unaffected; a nearly infinite number of sites for nonspecific binding exist relative to specific receptor(s). Thus, the amount of radioactivity remaining under these conditions is taken as a measure of nonspecific binding. It is calculated as follows:

Specific binding = Total binding – Nonspecific binding

where “Total binding” is the amount of radioligand bound in the absence of a competitor, and “Nonspecific binding” is that bound in the presence of an excess of competitor. The theory that is applied to the analyses of such data was discussed earlier.

19.3.6 The Relation Between Receptor Occupancy and Biological Response

The above discussion has focused on methods for describing the proximal events in receptor-mediated responses to toxicants, namely the binding of a toxicant ligand to a biochemically defined receptor. A critical assumption is that such an approach will elucidate the mechanisms by which receptor–toxicant interactions produce their ultimate toxic effects. The validity of this assumption rests on demonstrating that the binding of a ligand is related lawfully to some biological response. For instance, a straightforward prediction might be that the binding affinity of a toxicant for a receptor should correlate with its propensity to either stimulate or block a functional response. More often than not, however, this relation is complex rather than simple.

Stephenson is credited with introducing the concept of a complex relation between receptor occupancy and tissue response. His formulations were designed to explain prior anomalous data that suggested that drug effects were often not linearly related to receptor occupancy, as shown by the frequent finding that the maximal drug effect achieved was not linearly related to the concentration of drug applied. Stephenson introduced three postulates to explain such aberrant data. First, he stated that some agonists could produce a maximal response by occupying a small fraction of a receptor population. Second, he stated formally that the tissue response is not related linearly to receptor occupation. Finally, he introduced the concept of differing efficacies of agonists, such that an agonist with high efficacy may elicit a maximal effect when only a small percentage of receptors are occupied, while an agonist with low efficacy may require occupation of a substantially greater percentage of receptors. A partial agonist is a special case of a low efficacy agonist. That is, the efficacy of a partial agonist is so low that it fails to elicit a maximal response even at saturating concentrations, when all receptors presumably are occupied. The potential antagonistic effects of partial agonists present at high concentrations can now be appreciated; partial agonists can compete effectively for receptor binding sites with more efficacious ligands, although partial agonists have only minimal biological effects. Figure 19.8 provides an example of the functional response produced by a partial agonist and a full agonist. It should be noted that the com-
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Compounds in Figure 19.8 can completely compete for the receptor believed to mediate this functional response. Several distinct mechanisms may explain partial functional efficacy, and it is not always clear which are operative. It is important to remember that functional efficacy and affinity for the receptor (potency) need not correlate.

The phenomenon of partial agonism is but one example of the complexities encountered when relating binding data to biological response. Another noteworthy example is provided by the finding that many receptor populations appear to be larger than would be required to produce a maximal response. This phenomenon is referred to as “spare receptors” or, alternately, as “receptor reserve.” The strongest empirical data supporting this notion comes from studies using irreversible receptor inactivation which demonstrate that the maximum tissue response is not depressed until a large percentage of the receptor binding sites are eliminated.

Finally, it should be noted that a comparison of receptor binding data with functional response is inherently difficult. In the case of binding assays prepared from tissue homogenates, receptors are isolated from components that are involved in the ultimate cellular response; thus, the assays are conducted in artificially pure preparations. In contrast, functional assays typically require greater preservation of cellular integrity. This also means that receptor regulatory processes are more likely to be evident during functional assays. For instance, a common finding is that the response of an agonist decreases as a function of prior exposure to the same or a similar agonist; this phenomenon is referred to as homologous or heterologous desensitization, respectively. In some model systems (e.g., β-adrenergic receptor), one mechanism for desensitization involves internalization and sequestration of receptor molecules. Additionally, an intracellular enzyme (β-adrenergic receptor kinase; BARK), has been found to be activated by agonist stimulation; this kinase

Figure 19.8. Dose–response plot showing functional differences between partial and full agonist. These data illustrate that two compounds of similar potency may differ in functional efficacy. In this case, the full agonist causes the same degree of stimulation as does the endogenous ligand. Conversely, the partial agonist, although of similar potency, is only partially efficacious. These data are modified from actual published studies from the authors’ laboratory (Brewster et al. J. Med. Chem. 1990).
phosphorylates particular amino acid residues on the receptor which are involved in some aspects of the desensitized response. Clearly, such dynamic receptor regulation is a critical aspect of receptor–toxicant interactions, yet such processes are not likely to be captured in receptor binding assays using extensively washed membranes.

This preceding discussion was not intended to convince one that the study of receptor binding is of limited utility, but rather that it should be accompanied by an examination of functional endpoints, with a realization of the potential challenges (and rewards) involved in relating the two.

19.4 RELATIONSHIP OF RECEPTOR OCCUPANCY TO FUNCTIONAL EFFECTS

19.4.1 Classical Definitions of Receptor-Mediated Functional Effects

A common tenet of pharmacology is that ligands can be characterized by the nature of the functional effects elicited by their interaction with their target receptor. In a given assay, a ligand can be classified as a full agonist, partial agonist, neutral antagonist, or inverse agonist. This led to the theory of “intrinsic efficacy,” originally proposed as a measure of the stimulus per receptor molecule produced by a ligand. According to this notion, full agonists possess sufficiently high intrinsic efficacy such that they maximally stimulate all cellular responses linked to a given receptor. Partial agonists possess lower degrees of intrinsic efficacy (leading to submaximal responses), whereas inverse agonists reduce constitutive (ligand-independent, basal) receptor signaling. Neutral antagonists possess no intrinsic efficacy, but occupy the receptor to block the effects of full, partial, or inverse agonists. Thus, “intrinsic efficacy” referred to an invariant characteristic of a ligand—as opposed to the operational term “intrinsic activity,” which simply refers to the maximal effect (E\text{max}) of a ligand relative to a reference agonist in any given experimental system.

19.4.2 Evolving Concepts of Receptor-Induced Functional Changes

With the GPCRs in particular, it is now clear that a single receptor can signal through many signaling pathways, depending on the complement of G proteins and other signaling molecules associated with the receptor in a given cellular locale. As discussed in the previous section, the traditional view has been that a ligand will have the same functional characteristics at every function modulated by a single receptor. Thus, a full agonist would be expected to activate all of the signaling pathways linked to a receptor to the same degree, and a ligand that antagonizes one signaling pathway via a specific receptor should antagonize every pathway coupled to that receptor to the same extent.

It now seems clear that this premise is frequently incorrect. There has been a rash of data emerging within the past decade showing that certain ligands have quite diverse functional consequences mediated via a single receptor. Thus, it is clear that the classical concept of “intrinsic efficacy” as a system-independent constant, although once having conceptual utility, is probably not correct. Indeed, evidence that this was of importance is suggested by the fact that different research groups
coined unique names for essentially the same phenomenon (e.g., “functional selectivity,” “agonist-directed trafficking of receptor stimulus,” “biased agonism,” “protean agonism,” “differential engagement,” “stimulus trafficking,” etc.).

There are three reasons why this new concept [see Urban et al. (2006) for review] is of importance to molecular toxicology. In the narrowest sense, it may be critical to understanding the exact mechanism of some toxicants. In a broader sense, it certainly affects clinical toxicology and toxicological aspects of drug development. As an example, it has been shown recently that agonists for the serotonin 5-HT$_{2B}$ receptor can promote heart valvuopathies (e.g., as are caused by fenfluramine). Until the specific signaling pathway responsible for this effect is known, one sometimes may generate misleading hypotheses if a drug candidate is screened at a single functional pathway that is not the one mediating toxicity in the heart. By similar reasoning, this mechanism suggests that one may be able to do drug optimization by selecting compounds with differential effects at two signaling pathways mediated by the same receptor. Finally, for ligand-selective toxicants, risk assessment estimates can often be more precise if we know the affinity of a ligand for its receptor. Awareness of the target receptor’s key functional pathways, as well as the effects of the toxicant at each, should allow even greater predictive accuracy.

SUGGESTED READING

Cheng, Y. C., and Prusoff, W. H. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 percent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108, 1973. This is one of the most commonly cited, and least read, papers in the field.


Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B. K., Weinstein, H., Javitch, J. A., Roth, B. L., Christopoulos, A., Sexton, P., Miller, K., Spedding, M., and Mailman, R. B. Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.* **2006**. www.graphpad.com. This is the website of the company that markets Prism, the most widely used software for receptor-ligand analyses. There is a wealth of background information offered free by this company (see Resource Library on home page) that can help even experienced scientist avoid common mistakes.
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