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Neurotransmitter Receptor Binding

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Preface

The use of ligand-binding techniques to study neurotransmitter receptor sites has led to a broad range of important biological advances. It is now possible to obtain information about the regional distribution of neurotransmitter receptors in brain, the pharmacological, biochemical, and developmental characteristics of these sites, and the functional interrelationships between neuronal cell types. In addition, the receptor-binding procedure has been used as an assay technique to measure levels of neurotransmitters and drugs, to identify new transmitter candidates, and to separate and purify receptors. The simplicity and versatility of these techniques render them attractive biochemical tools for routine use in neuroscience laboratories.

As with all methods, it is necessary to understand some of the basic principles of the technique in order to use a receptor-binding procedure appropriately. To this end, this text covers the fundamental principles of neurotransmitter receptor-binding assays, discusses the precise methodology for some of the more commonly used assays, and describes ways in which the assays can be used in neurobiological investigations, drug screening, neurochemical and histochemical analysis, and in the solubilization and isolation of receptors. This book is not intended as a review of the literature in these areas, but rather as a guide to enable neuroscientists, from technicians to senior investigators, to understand the basis of receptor-binding assays and to help establish these procedures in the laboratory. The utility and limitations of the binding assays in industrial drug screening, clinical medicine, and basic research are stressed.

Our chief goal has been to provide the reader with fundamental principles and procedures. Discussions of specific studies are limited to those that illustrate and clarify these principles. By using this approach, we hope that this book will serve not only as a laboratory guide but also as a catalyst for future investigations.

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Overview of Neurotransmitter Receptor Binding

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New areas of science often develop following the introduction of a novel technique. Such advances need not be particularly profound in terms of technological complexity. More frequently, the availability of new simple, sensitive, and specific procedures attracts the interest of large numbers of investigators with a resulting escalation in the rate of new findings. In psychopharmacology, the introduction during the mid-1950s of chemical assays for norepinephrine and serotonin that were sensitive, specific, and relatively simple sparked a vast body of research into the role of these substances in the brain and almost literally initiated the field of biochemical psychopharmacology. Relatively simple procedures for preparing fractions of brain tissue enriched in pinched-off nerve terminals or synaptosomes greatly accelerated the investigation of numerous chemicals as neurotransmitter candidates. The availability of radiolabeled biogenic amines and their precursors at high specific activity prompted investigations of transmitter uptake and turnover. Straightforward biochemical assays measuring tyrosine hydroxylase activity permitted the discovery of α-methyl-*p*-tyrosine and other agents as inhibitors of the enzyme, permitting analyses of catecholamine turnover and the regulation of tyrosine hydroxylase activity.

Neurotransmitter receptors may in most instances consist of at least two distinct components, the recognition or binding site and some second portion of the receptor which "translates" transmitter recognition into a second messenger, usually an alteration in ion permeability or accumulation of a cyclic nucleotide. The bulk of this book is devoted to the recognition site of neurotransmitter receptors, an area in which research has increased dramatically in recent years.

The notion that drugs and biologically active compounds act at specific receptor sites has been accepted since the turn of the century. In the case of neurotransmitters, much early evidence derived from neurophysiological investigations. Biochemical approaches to receptors lagged behind neurophysiological investigations and behind almost all biochemical research into synaptic events. By 1970 the biosynthesis, storage, release, and inactivation of numerous transmitters had been characterized biochemically while little was known of receptors at a

biochemical level. Major problems involved specificity and sensitivity in assays detailed as follows. The most straightforward approach to identifying receptor sites would be to measure the binding of the radioactive transmitter or a related drug to membranes from an appropriate target organ. However, most transmitter candidates and drugs possess hydrophilic and hydrophobic portions which can interact by ionic as well as non-ionic means with numerous sites on biological membranes. The numbers of such nonspecific binding sites would be expected to exceed vastly the minute number of specific transmitter receptors. The problem of sensitivity arises for ligands with high receptor affinity. Unless radiolabeled forms of the ligand have a high enough specific activity to permit assay at concentrations as low or lower than the dissociation constant, the concentration of radioactive ligand which must be added to produce measurable radioactivity in the membranes may saturate specific receptors and label primarily nonspecific sites.

Paton and Rang (39) provided a pioneering paradigm for labeling neurotransmitter receptors in studies evaluating binding of ^{3}H-atropine to slices of guinea pig ileum. Although most of the binding involved nonspecific sites, a portion labeled muscarinic receptor sites. Because of the low specific activity of the ^{3}H-atropine employed, the receptor was not characterized in great detail and these elegant observations were not pursued on a large scale. Subsequently the reversible anticholinergic ^{3}H-quinuclidinyl benzilate has proved valuable for efficient labeling of muscarinic receptors with selectivity and low levels of nonspecific binding (60).

Rang and co-workers (16,19) subsequently employed muscarinic drugs with alkylating mustard properties as receptor labels. The shift from reversible to irreversible ligands by these workers reflects an ongoing debate between exponents of the two approaches. Alkylating agents may form potentially irreversible covalent links to receptor sites that can be valuable in attempts to monitor the receptor during solubilization and purification. However, the alkylated receptor usually lacks biological activity. Since the goal of solubilization and purification is to examine transmitter interactions with the receptor, alkylating the receptor may be akin to "cutting off your nose to spite your face." A potentially more serious problem relates to specificity. With reversible ligands receptors can be labeled selectively if the ligand has higher affinity for the receptor than for nonspecific binding sites. However, with incubations of sufficient duration an alkylating agent labels sites of low as well as of high affinity. One of the most successful uses of alkylating agents to label neurotransmitter receptors involves benzilyl choline mustards developed by Rang and co-workers (16,19) and subsequently by Burgen and his colleagues (5). The latter group utilized these mustards for autoradiographic visualization of the receptors, a task for which irreversible ligands are better suited than reversible ones

Although historical priority may belong to studies of the muscarinic cholinergic receptor, the application of binding techniques to the nicotinic cholinergic receptor in 1970 attracted more scientific visibility and drew many scientists

into the investigation of neurotransmitter receptors. A crucial ingredient in these studies was the ligand α-bungarotoxin. This polypeptide is a potent and almost irreversible antagonist at nicotinic cholinergic receptors. Using ^{125}I-labeled α-bungarotoxin, several workers (7,30,32,45) identified specific binding to nicotinic cholinergic receptor sites in membranes of the electric organ of the electric fish. α-Bungarotoxin was shown to have a dissociation constant in the nanomolar range and to dissociate only very slowly from binding sites. Nicotinic agonists and antagonists competed for binding with potencies which in general corresponded to their pharmacological activity whereas nonnicotinic agents lacked activity. Because α-bungarotoxin dissociated so slowly from receptor sites, it was possible to solubilize the nicotinic receptor with detergents such as Triton-X-100 and to purify the receptor extensively. In early studies it was shown that the bungarotoxin binding protein was a physically distinct entity from acetylcholinesterase, which refuted earlier hypotheses that these were both parts of the same macromolecule.

Even prior to the α-bungarotoxin investigations, O'Brien and associates had demonstrated binding interactions involving nicotinic cholinergic receptors in electric organs using ^{3}H-muscarone and assaying the binding by centrifugation (37) and equilibrium dialysis (38). The difficulty with equilibrium dialysis as a tool to detect binding interactions is that one measures a reduction in radioactivity of the ligand in the incubation medium so that small amounts of binding are not readily measured. Karlin and Cowburn (25) utilized unique nicotinic ligands which formed covalent associations with the receptor through sulfhydryl groups obtained after reduction of a disulfide bond of the receptor with dithiothreitol. Specificity was ensured by regarding as "specific" interactions only those which were prevented by omitting dithiothreitol.

Earlier studies of hormone receptors provided a major impetus to work with neurotransmitter receptor sites. The classic studies of Jensen and Jacobson (23) beginning in 1962 identified estradiol receptors. Jensen first demonstrated these receptors in intact animals by showing that target organs such as the uterus, vagina, and pituitary accumulated ^{3}H-estradiol in a saturable fashion which could be antagonized by steroids in proportion to their estrogen-like activity. A technical breakthrough making Jensen's studies feasible was his own synthesis of ^{3}H-estradiol of very high specific radioactivity. This was important because estrogen receptors have extremely high affinity for their hormone. Subsequently Jensen et al. (24), Gorski et al. (21), and other workers showed that estrogen and other steroid hormone receptors are cytoplasmic and move to the nucleus following interactions with their hormone. By contrast, neurotransmitter receptors are thought to be localized primarily to the external surface of neuronal membranes.

Receptors for peptide hormones historically have provided a more useful model for neurotransmitter receptors. Indeed it is now becoming evident that several peptide hormones may also serve neurotransmitter functions.

Studies of the insulin receptor by Cuatrecasas (11) and Roth and colleagues

(18) provided valuable information about receptor properties and technological approaches. In the case of peptides such as insulin, great care must be taken to ensure that labeling the hormone with ^{125}I does not impair biological activity. Overly rigorous iodination may add more than one iodine per insulin molecule, which interferes with biological and binding activity. Some studies of the insulin receptor have highlighted questions of specificity. For instance, it was shown that ^{125}I-insulin could bind with high affinity to talcum powder, and that the relative potencies of some insulin analogs in competing for binding paralleled their biological activity (12). Clearly numerous agents must be examined critically before concluding that the biologically meaningful receptor is being studied. In the case of insulin direct comparisons can be made between binding and physiological activity by measuring the oxidation of glucose as well as binding in intact fat cells. For central neurotransmitters the brain does not usually provide simple biochemical assays of physiological activity.

To detect specific receptor binding and to minimize nonspecific binding required technical innovations. In measuring insulin receptors Cuatrecasas assayed membrane-bound insulin by filtration with a manifold which permits the analysis of many samples simultaneously and an efficient vacuum which facilitates rapid but thorough washing to remove nonspecifically bound ligand without perturbing receptor-bound compound. These relatively simple technological principles have been of considerable importance for studies of central neurotransmitter receptors.

The logical sequence of events in neuropharmacology begins with a known neurotransmitter whose receptor interactions are characterized, and finally potential receptor-active drugs are evaluated. In the case of the opiate receptor this sequence was reversed. A historical description of efforts to characterize the opiate receptor illuminates some important methodological and theoretical issues. It had been well known that most pharmacological activities of opiates are stereospecific with the (−)-isomer possessing much greater potency than the (+)-isomer. Since the late 1950s numerous workers have attempted to measure the binding of radioactive opiates to brain membranes, using as their screen for selective interactions with the opiate receptor stereospecificity of displacement of binding by plus and minus isomers. In 1971 Goldstein et al. (20) noted that ^{3}H-levorphanol bound to membranes in mouse brain homogenates and that approximately 2% of the binding could be displaced stereospecifically by levorphanol but not by dextrorphan, the pharmacologically inactive isomer. The minute amount of stereospecific binding precluded detailed characterization. The specific activity of the ^{3}H-levorphanol employed was quite low so that high concentrations, approximately 0.1 mM, were added routinely. Because binding sites of high affinity would have been completely saturated at this concentrations, the binding sites evaluated by Goldstein et al. (20) would have had to possess relatively low affinity. Another puzzling aspect of these sites was their localization to nuclear fractions of brain homogenates and the fact that highest densities of the binding sites occurred in areas of the brain rich in white matter. Subsequently Goldstein succeeded in amplifying the amount of stereospecific

binding and in solubilizing and purifying the binding substance. Loh et al. (29) then showed that the binding material did not contain protein but consisted almost exclusively of cerebrosides. Subsequently Abood and Hoss (1) showed that a variety of acidic lipids could bind opiates stereoselectively. Indeed even synthetic filters used in some binding studies bind radioactive opiates stereoselectively with the (−)-isomer being more potent than the (+)-isomer (51).

This story illustrates the importance of a detailed and rigorous demonstration of specificity of receptor interactions, preferably with comparisons of binding and biological activity in the same tissue. Thus the subsequent demonstration of high-affinity stereospecific binding of ^{3}H-opiates to brain membranes (41, 47,53) was a necessary but not sufficient criterion for identification of the opiate receptor. A correlation of binding potencies with analgesic activity provided further evidence. Most conclusive evidence derived from comparisons of binding affinities in membranes from the guinea pig ileum with pharmacological activities of opiate agonists and antagonists in the same tissue (8).

Besides providing lessons about technical approaches to receptor binding, the opiate receptor has afforded insights into the differences between agonist and antagonist interactions with neurotransmitter receptors and how recognition of the transmitter might be translated into changes in ion permeability. When assays were conducted with Tris buffers, agonists and antagonists did not differ in their affinities for binding sites (42). However, small concentrations of sodium markedly enhanced the binding of ^{3}H-opiate antagonists while diminishing the binding of ^{3}H-agonists (44). The effect of sodium was potent, occurring at 1 mM sodium, and selective, being manifested to a limited extent by lithium but not rubidium, cesium, or potassium (43). These findings were consistent with a model of the opiate receptor in which two interconvertible states prefer agonists and antagonists, respectively. The pharmacological properties of an antagonist occur because it binds to the "antagonist" state of the receptor, making fewer "agonist" states available for opiates or opioid transmitters. One can postulate that the ion whose permeability is relevant to transmission, in this case sodium, binds preferentially to one of the two states of the receptor, in this case the antagonist state. Normally receptor sites are bathed in sodium. By binding to the agonist state of the receptor, morphine would provoke a transformation of antagonist conformations of the receptor to agonist conformations, "loosening" the binding of sodium and thus altering its membrane permeability. A direct role of sodium has been demonstrated in mediating neurophysiological inhibitory influences of opiates and opioid peptides (63).

Interactions of the ion conductance modulator and receptor binding sites have also been apparent in studies of the glycine receptor. Glycine is thought to be a major inhibitory neurotransmitter in the spinal cord and lower brainstem. Glycine receptors can be labeled with the glycine antagonist ^{3}H-strychnine (61). Synaptic hyperpolarization elicited by glycine involves an increase in chloride conductance. Physiological concentrations of chloride reduce the binding of ^{3}H-strychnine and also decrease the affinity of glycine for the binding sites

(36,62). The ability of 14 anions to mimic this effect of chloride corresponds closely with their relative abilities to mimic the reversal of inhibitory postsynaptic potentials by chloride. Whether these binding interactions distinguish glycine agonists and antagonists has not been adequately investigated because it has not proved possible to label glycine receptors with glycine itself or another agonist.

Besides glycine, the other major inhibitory neurotransmitter in the brain is also an amino acid γ-aminobutyric acid (GABA). In contrast to the glycine receptor, which is best labeled with the antagonist strychnine, the GABA receptor has been most effectively studied by the binding of the agonist ^{3}H-GABA (64) or ^{3}H-muscimol (2,15). Recent studies of the GABA receptor with the antagonist ^{3}H-bicuculline (33) suggest that it may label a state of the receptor with preferential affinity for antagonists. However the apparent affinities of agonists and antagonists for sites of the GABA receptor labeled by ^{3}H-GABA and ^{3}H-bicuculline, respectively, do not differ as markedly as is the case for certain other receptors.

A prime candidate for the major excitatory transmitter in the brain is also an amino acid, glutamic acid. The evidence for glutamic acid as a transmitter is weaker than is the case for other amino acid transmitters, such as glycine or GABA. Kainic acid, a rigid analog of glutamic acid, is substantially more potent than glutamate in causing synaptic excitation. Binding sites labeled by ^{3}H-kainic acid exhibit a substrate specificity suggesting that the binding sites represent the physiological glutamate receptors (48).

Interactions between recognition and "second messenger" sites of a transmitter receptor have also been identified for β-noradrenergic receptors. In this case, the second messenger appears related to adenylate cyclase rather than to a specific ion. β-Receptors have been successfully labeled with ^{3}H-dihydroalprenolol (^{3}H-DHA), ^{3}H-propranolol, and ^{125}I-hydroxybenzylpindolol (^{125}I-HYP). Recently it has been possible to label β-receptors in some tissues with agonists such as ^{3}H-hydroxybenzylisoproterenol (28) and ^{3}H-epinephrine (55). Physiological desensitization of β-receptors by chronic exposure to catecholamines in frog erythrocytes is reflected in decreased responsiveness of the adenylate cyclase to stimulation by catecholamines. Desensitization is accompanied by a reduction in ^{3}H-DHA binding and a slowing of the dissociation of the agonist ^{3}H-hydroxybenzylisoproterenol associated with a higher affinity of the agonist (28,31,35). An increased affinity of transmitter for receptors had been postulated as the mechanism of desensitization at nicotinic receptors of the neuromuscular junction by Katz and Thesleff on physiological grounds (26). Enhanced agonist affinity at nicotinic receptors in preparation of electric organs during desensitization has also been demonstrated by Weber et al. (59). It is conceivable that such desensitization is a universal aspect of neurotransmitter receptor interactions. In any event, Mukherjee and Lefkowitz showed that guanyl nucleotides can reverse the physiological desensitization and simultaneously accelerate the dissociation of ^{3}H-hydroxybenzylisoproterenol from β-receptors (35). Under

normal circumstances GTP and related nucleotides decrease the affinity of β-agonists for receptor sites but only in tissues that can be physiologically desensitized (e.g., frog but not turkey erythrocytes).

Distinct binding states for agonists and antagonists have been clearly demonstrated with dopamine and α-norepinephrine receptor in the brain. ^{3}H-Dopamine and ^{3}H-apomorphine appear to label agonist states of the dopamine receptor, whereas ^{3}H-haloperidol and ^{3}H-spiroperidol label antagonist preferring states (6,10,17,46). Thus dopamine agonists have substantially greater potency in competing for the binding of ^{3}H-dopamine than ^{3}H-haloperidol, and the reverse holds for dopamine antagonists. Agonist states of α-noradrenergic receptors can be labeled with ^{3}H-clonidine, ^{3}H-epinephrine, and ^{3}H-norepinephrine, and antagonist states can be labeled with ^{3}H-WB-4101, and ^{3}H-dihydroergokryptine behaves in an intermediate fashion (56–58). Whereas agonist and antagonist states of opiate and dopamine receptors appear to be interconvertible, the two binding states of the α-receptor are apparently distinct receptors. Although α-receptors are not traditionally thought to be associated with adenylate cyclase, influences of GTP and related nucleotides on α-receptor binding are essentially the same as on β-receptors in tissues in which desensitization can be demonstrated. Thus GTP diminishes the potencies of α-agonists and accelerates their dissociation from receptor sites.

Serotonin synaptic receptors can be labeled with the transmitter ^{3}H-serotonin (4). The binding of ^{3}H-LSD to brain membranes also has properties of serotonin receptors in most parts of the brain (3,4). Relative affinities of serotonin agonists, antagonists, and ergots suggest that ^{3}H-LSD behaves as a mixed agonist–antagonist in labeling serotonin receptors (4). In the corpus striatum, which is rich in dopamine neurons, approximately 20% of ^{3}H-LSD binding involves dopamine receptors while the majority remains associated with serotonin receptors. The possibility that a radioactive ligand can label a multiplicity of receptor sites is highlighted by studies of ^{3}H-dihydroergokryptine (^{3}H-DHE). In the rabbit uterus, ^{3}H-DHE binding appears exclusively associated with α-noradrenergic receptors (58). In the brain low concentrations of ^{3}H-DHE also selectively label α-receptors (22). However, at slightly higher concentrations (5 to 10 nM) ^{3}H-DHE labels serotonin as well as α-receptors in most brain regions (13), and in the corpus striatum ^{3}H-DHE labels dopamine receptors as well (54). Thus a single ligand can label three receptor sites in the brain. These findings are in accord with the pharmacological data that ergots such as DHE have potent effects at dopamine, α- and serotonin receptors.

Interestingly, the β-receptor does not seem to distinguish agonists and antagonists. Agonists and antagonists have similar potencies in competing for binding of agonists ^{3}H-hydroxybenzylisoproterenol and ^{3}H-epinephrine as in the binding of antagonists such as ^{3}H-DHA (27,28,56).

One of the most recent brain receptors to be identified is the benzodiazepine receptor (34,52). The relative potencies of benzodiazepines in competing for high-affinity binding of ^{3}H-diazepam closely parallels their pharmacological po-

tencies. No other drugs or neurotransmitters examined have any substantial affinity for these binding sites. In analogy with the opiate receptor, it has been proposed that there may be some normally occurring benzodiazepine-like neurotransmitter.

Neurotransmitter receptor binding has led to a number of practical applications in industry as well as in research laboratories, some of which are detailed in later chapters of this book. Opiate and dopamine receptor binding assays are used routinely for screening potential analgesics and antischizophrenic drugs, respectively. Affinities of neuroleptics for muscarinic cholinergic (50) and α-noradrenergic receptors (40) predict extrapyramidal and sedative side effects of neuroleptics, respectively. The clinically important anticholinergic effects of tricyclic antidepressants and neuroleptics are predicted by their affinities for muscarinic cholinergic receptor binding (49).

Another practical application involves the use of receptor binding as a tool to measure drugs or neurotransmitters themselves. Such a radioreceptor assay for GABA (14) is at least as sensitive and specific as any other known assay and considerably simpler to perform. A radioreceptor assay for neuroleptic drugs has several virtues over other analytical procedures (9). Since the therapeutic effect of these drugs in schizophrenic patients derives from blockade of dopamine receptors, the assay can detect any of the large number of neuroleptic drugs marketed. Moreover, it monitors active metabolites as well as the parent drug.

In summary, in the course of a relatively few years studies of neurotransmitter receptors by binding techniques have made considerable advances. The most generally applicable approach to labeling receptors has been to utilize reversible ligands, either the transmitters themselves or appropriate agonist or antagonist drugs. Using these procedures receptor sites for almost all the known neurotransmitters in the peripheral and central nervous system can now be monitored biochemically. Binding assays have permitted an approach to relating recognition of the transmitter to some second messenger involving ion conductance changes or cyclic nucleotide alterations. Assays of neurotransmitter receptor binding have had practical ramifications in the design of new drugs and in radioreceptor assays.

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