Drug and Neurotransmitter Receptors in the Brain

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Summary. Biochemical investigation of receptors for neurotransmitters and drugs in the brain has been one of the most active areas of molecular neuroscience during the past decade. This work has permitted fundamental insights into how binding of neurotransmitters to their receptors excites or inhibits neuronal firing or changes cellular metabolism. The recognition of receptor subtypes has suggested subtle ways for neurotransmitters to modulate neuronal functioning. Finally, the ability to measure receptor sites in simple test tube systems and to distinguish readily between agonists and antagonists has provided useful probes for drug discovery programs.

Synapses are the nodal points for information in the brain. Information is processed by the transfer of excitatory or inhibitory impulses among the 10 billion or more neurons in the human brain. In almost all instances synaptic transmission involves the passage of chemicals called neurotransmitters from endings of one neuron to an adjacent neuron. Somehow recognition of the neurotransmitter at the neuronal membrane surface alters ion permeability or metabolism and thence firing rates.

Specific enzymatic steps or precursor accumulating mechanisms regulate the biosynthesis of the transmitter. Neurotransmitters are generally stored in synaptic vesicles, and specialized release mechanisms govern their egress from the nerve ending by "exocytosis," a mechanism in which the membrane of the vesicle is thought to fuse with the neuronal membrane. Neurotransmitters are inactivated rapidly after they influence other cells by enzymatic degradation or pump-like accumulation into the neuron that had released them or into other neurons or glia, the supporting cells of the nervous system. Finally, neurotransmitters interact with specific recognition sites on adjacent neurons. These recognition sites, designated receptors, are proteins located on the membranes of adjacent neurons and sometimes on glial cells. The term "receptor" is sometimes used to designate the totality of events whereby recognition of a neurotransmitter alters cellular function. It is also used in a more narrow sense, referring to the specific membrane protein that recognizes and binds the neurotransmitter. This article deals primarily with receptors as recognition sites, although an important theme is how recognition of the neurotransmitter is "translated" into altered cellular function.

Neurotransmitter synthesis, storage, release, degradation, and inactivation by uptake mechanisms have been extensively characterized. Molecular studies of neurotransmitter receptor recognition sites in the brain have occurred mostly during the past 10 years. Studies of receptor recognition sites have been made possible by the development of simple and sensitive techniques to measure the binding of radioactive drugs or neurotransmitter molecules to brain membranes. As early as 1965 Paton and Rang (*1*) monitored the uptake of radioactive atropine by intestinal muscle, which appeared to involve acetylcholine receptors. The first widely exploited strategy for measuring receptor binding came from studies of the acetylcholine receptor of electric organs of invertebrate fish such as electric eels (*2*). In these electric organs the acetylcholine receptor comprises as much as 20 percent of membrane protein. Very potent and virtually irreversibly binding toxins such as α-bungarotoxin were labeled with ^{125}I to identify the receptors. By contrast, one could calculate that, in the brain, most neurotransmitter receptors would represent no more than about one-millionth by weight of brain tissue, and no selective, potent toxins seemed available. With so few receptor sites and the availability only of reversibly binding drugs, isotopically labeled binding agents would be expected to interact nonspecifically with many membrane proteins, lipids, and carbohydrates. The "noise" of such nonspecific binding might vastly exceed the minute signal from specific receptor interactions.

Success in measuring drug and neurotransmitter receptor recognition sites in the brain stemmed from rapid filtration techniques and extensive but rapid washing, simple techniques that permitted the removal of the labeled group from nonspecific sites while leaving receptor-bound ligands intact. Such techniques were applied first to the opiate receptor (*3–5*), but were subsequently used with a wide range of receptor sites in the brain, including receptors for the inhibitory amino acid neurotransmitter glycine (*6*), muscarinic acetylcholine receptors (*7*), γ-aminobutyric acid (GABA) receptors (*8*), serotonin receptors (*9*), dopamine receptors (*10*), and α-adrenergic (*11*) and β-adrenergic receptors (*12*).

Studies of ligand binding to tissue membranes have often been accompanied by announcement of newly identified "receptors." However, all sorts of chemical substances can appear to bind with a high degree of affinity and specificity to biological and even nonbiological membranes when in fact they only bind nonspecifically. Thus it is important to ascertain specificity in any study of this kind. For example, we were able to identify labeled opiate binding to glass filters stereospecifically and in the same direction as the stereospecificity of opiate receptors (*13*). Insulin binding to talcum powder has been described which mimics many properties of insulin receptors (*14*). To establish that one is dealing with a biologically relevant receptor site, it is important to show that relative potencies of numerous agents at the binding site correlate with their biological effects. In the case of the opiate receptor we took advantage of the well-known ability of opiates to inhibit electrically induced contractions of intestinal smooth muscle, an effect which correlates with analgesic potencies. We measured the influence of opiate agonists and antagonists on contractions of guinea pig intestine and measured the relative potencies of the same drugs at binding sites in the same intestinal preparations (*15*). The close correlation between pharmacological and binding potencies of these agents ensured that the binding

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sites represented pharmacologically relevant opiate receptors.

The ability to label receptor sites in simple binding systems has provided a means for examining a number of basic questions. One can explore the relation of the receptor recognition protein to macromolecules regulating ion permeability or to second messenger formation. For instance, the inhibitory synaptic actions of glycine are mediated by an increase in neuronal membrane permeability to chloride ion. The binding of the glycine antagonist [^{3}H]strychnine to glycine receptors is modulated by physiological concentrations of chloride, and the relative potencies of various anions in eliciting this effect parallel closely their relative abilities to mimic the synaptic hyperpolarization elicited by chloride (*16*). For neurotransmitters whose actions are mediated by adenylate cyclase [the cyclic adenosine monophosphate (AMP)–forming enzyme], guanosine triphosphate (GTP), acting through a specific GTP binding protein, links the neurotransmitter recognition site to adenylate cyclase. Binding sites for several neurotransmitters that act via adenylate cyclase are modulated by physiological concentrations of GTP (*17*). In some instances the apparent link of the receptor recognition protein to the GTP binding protein is maintained even after the receptor is solubilized from neuronal membranes (*18*). It is now possible to study detailed molecular interactions between receptor recognition proteins, GTP binding proteins, and the adenylate cyclase molecule (*19*).

Virtually every drug that alters mental function does so by interacting with a neurotransmitter system in the brain. Many drugs mimic or block the effects of specific neurotransmitters at receptor sites. Measuring receptors in simple binding assays has proved a powerful tool for drug discovery programs. Prior to the advent of such molecular techniques for drug screening, most drug evaluation had been carried out in intact animals. Using receptor binding techniques, the chemist may synthesize only a few milligrams of the chemical. Various drug potencies will be related only to variations in receptor affinity, facilitating systematic structure modification to obtain more potent agents. Subsequent bioavailability studies may augment in vivo activity. One example of a receptor approach to drug development involves xanthines, which include caffeine as well as the major antiasthmatic drug theophylline. Xanthine drugs exert some of their pharmacologic actions by blocking receptors for adenosine. Systematic modifications of the xanthine structure along with parallel adenosine receptor assays resulted in derivatives up to 500,000 times more potent in blocking receptors than caffeine (*20*).

Obtaining more selective therapeutic agents has been facilitated by studies of multiple neurotransmitter receptor subtypes (*21*) (Table 1). Classic pharmacologic studies have revealed more than one receptor for certain neurotransmitters. Thus, acetylcholine acts at distinct nicotinic and muscarinic receptors. Norepinephrine effects are mediated via α or β receptors, and conventional pharmacologic analysis has differentiated β receptors into β_1 and β_2 types. Monitoring receptors in binding studies has enhanced discriminative subtlety so that a greater multiplicity of receptor subtypes can be identified. For instance, binding studies of opiate receptors differentiate at least three subtypes of receptors: μ, δ, and κ (*22*). As many as four different types of dopamine receptor binding sites have been reported (*23*). The α_1 and α_2 adrenergic receptors can be discriminated, and distinct high and low affinity types of α_2 receptors can be monitored (*24*). Binding studies have revealed at least two subtypes of serotonin receptors (*25*).

Receptor multiplicity provides a way for neurotransmitters to influence cells in subtle ways. It also makes possible the design of more selective drugs. There is evidence that μ opiate receptors are largely involved in the analgesic effects of opiates, where δ opiate receptors relate more to emotional effects of the drugs, and κ sites may mediate the sedative effects of certain opiates (*22, 26*). Clearly, receptor-selective opiates might have fewer adverse side effects. Similarly, subtypes of benzodiazepine receptors may be differentially involved in the sedative and antianxiety actions of these drugs (*27*).

The existence of numerous distinct neurotransmitters also provides multiple sites for the actions of drugs. One of the striking themes of modern neuroscience research has been that the brain has many more neurotransmitters than had been previously supposed. The major chemical classes of neurotransmitters are amines, amino acids, and peptides. The number of peptide neurotransmitter candidates has escalated considerably in the past few years. Forty or more distinct neuropeptides may serve as transmitters. The neuronal localizations of most of these are just as selective and possibly relevant to mental disease as the classical biogenic amine neurotransmitters.

Understanding the functions of receptors has been enhanced by the convergence of neuroanatomical and molecular techniques in autoradiographic visualization of neurotransmitter receptors based on the work of Kuhar (*28*). Thin brain slices are incubated with the radioactive ligand under conditions in which the drug is selectively bound to receptor sites with negligible nonspecific binding. Silver grains related to receptor-bound ligand are visualized after development with photographic emulsion on tritium-sensitive film. This approach has proved effective for elucidating mechanisms whereby drugs exert their pharmacologic actions in the brain. For instance, the autoradiographic localization of opiate receptors clarified many of the pharmacologic actions of these drugs (*29*). Discrete localizations of opiate receptors occur in specific areas associated with pain perception and emotional regulation, explaining analgesic and euphoric actions of opiates. Localizations of histamine H_1 receptors in the vestibular area of the brainstem can explain how antihistamines relieve motion sickness (*30*).

While receptor autoradiography is now most efficiently carried out by labeling the receptors in vitro in brain slices, for initial autoradiographic studies ^{3}H-labeled drugs were administered in vivo under conditions in which most of the labeled drug in the brain was associated with receptor sites. In vivo labeling of receptors has now been applied (with appropriate isotopes) in humans to visualize dopamine receptors by positron emission tomography (PET scanning) (*31*). Potential receptor abnormalities in disease states can be evaluated by this approach. In vitro autoradiographic examination of receptors in postmortem human brain tissue has proved useful for studies in neuropathology (*32*).

Opiate Receptors

Opiate receptor research has exemplified a number of themes, including autoradiographic visualization of receptors, investigation of multiple receptors, and elucidation of how receptor recognition alters cellular function. The differentiation of agonists and antagonists is fundamental to pharmacology. A neurotransmitter is defined as having agonist activity, while a drug that blocks the actions of the neurotransmitter is an antagonist. Morphine and related opiates are agonists, and drugs that block the effects of opiates are designated antagonists.

Understanding why drugs differ along an agonist-antagonist continuum may

provide insight into how receptor recognition changes cellular function. By definition, receptor recognition of an agonist is "translated" into other cellular alterations, whereas an antagonist occupies the receptor without initiating the transduction step. There are therapeutic reasons for our wanting to understand differences between agonists and antagonists, especially in the opiate field. It has been recognized for many years that drugs combining opiate agonist and antagonist activity can relieve pain while having a smaller addictive potential than pure agonists such as morphine. The pharmaceutical industry has tried to identify such mixed agonist-antagonists. However, conventional screening techniques in intact animals had only limited efficacy in identifying such agents.

In early studies of opiate receptors, agonists and antagonists could not be distinguished from one another. The antagonist nalorphine differs from its related agonist morphine only in that nalorphine possesses an *N*-allyl substituent which in morphine is an *N*-methyl group. In sodium-free buffers, morphine and nalorphine displayed identical affinities for opiate receptors. In our first evaluation of ion effects on opiate receptors, sodium did not affect the binding of the antagonist [^{3}H]naloxone (*33*), whereas Simon *et al.* found reduced binding of the agonist [^{3}H]etorphine in the presence of sodium (*5*). We then observed that sodium ion systematically differentiates opiate agonists and antagonists (*34*). The receptor affinity of pure antagonists such as naloxone is unaffected by sodium, while pure agonists become up to 50 times weaker in the presence of sodium. The mixed agonist-antagonists that offer potential as less addicting analgesics are three to seven times less potent in the presence of sodium ion. This effect of sodium is potent and selective, not being manifest with other monovalent cations such as potassium, rubidium, or cesium (*35*).

The effect of sodium on drug interactions with opiate receptors is now used routinely in the pharmaceutical industry for screening opiates on an agonist-antagonist continuum. These findings suggested that sodium ion plays a role in relating receptor recognition to altered cell function. One possibility was that sodium ion permeability might be altered when opiates interact with receptors (*35*). It now appears more likely that sodium, together with GTP, influences interactions of the opiate receptor with a GTP binding protein and adenylate cyclase (*36*).

Like sodium, GTP and other guanine nucleotides differentiate agonists and antagonists, inhibiting receptor binding of agonists but not antagonists and influencing mixed agonist-antagonists. Sodium and GTP are synergistic in these effects, so that the maximum differentiation of agonists and antagonists is obtained by a combination of sodium and GTP (*37*). The influence of GTP on opiate receptor binding fits well with evidence, first obtained in neuroblastoma-glioma cells in culture, that opiates act through adenylate cyclase (*38*). Opiate agonists reduce adenylate cyclase activity of these cells.

Opiates provide a useful paradigm for receptor multiplicity. On the basis of the different pharmacologic effects of numerous drugs, Martin *et al.* (*39*) postulated several subtypes of opiate receptors. The μ receptors mediate effects of morphine-like opiates that also produce euphoria. Drugs with κ activity are analgesic and sedative but elicit less euphoria and are less addicting than morphine. Another receptor, designated σ, was postulated to account for the psychotomimetic effects observed with some opiate drugs, particularly mixed agonist-antagonists.

Receptor binding studies have extended the work of Martin *et al.* Binding sites for the psychotomimetic drug phencyclidine have almost the same affinity for psychotomimetic opiates as phencyclidine, but nonpsychotomimetic opiates are inactive (*40*). Thus, the σ receptor may not be an opiate receptor at all but a site at which phencyclidine acts, although recent work indicates a specific σ, psychotomimetic opiate receptor distinct from the phencyclidine receptor (*41*). The failure of opiate antagonists to reverse the behavioral effects of psychotomimetic opiates supports the notion that such effects do not involve conventional opiate receptors (*42*).

Using labeled drugs that were suggested by Martin as having κ activity, Kosterlitz and associates identified κ receptor binding sites (*43*). Kappa receptor activity can also be assessed by selective effects on contractions of rabbit vas deferens (*44*). Kosterlitz and colleagues (*45*) also identified a new subclass of opiate receptors called δ receptors. The μ and δ receptors were distinguished both by binding studies and by their differential effects on contractions of smooth muscle. Contractions of mouse vas deferens are influenced fairly selectively by δ-specific drugs, while guinea pig intestine responds more to μ agents. Morphine is 50 to 100 times weaker at δ than at μ receptors. By contrast, the endogenous opiate-like neurotransmitter peptides known as enkephalins tend to be more potent at δ than μ receptors. Receptor subtypes vary in the same organ of different species. Thus, the rat vas deferens has an ϵ receptor that is selective for the opioid peptide β-endorphin (*46*).

In receptor binding studies, a distinctive, high affinity site marked by all [^{3}H]-labeled opiates examined has been designated μ_1 (*47*). Naloxonazine, a long-acting naloxone derivative, selectively blocks μ_1 sites and thus permits an analysis of their functional role. The μ_1 receptors are implicated in analgesic but not respiratory depressant effects of opiates. Thus, μ_1 selective opiates might be safer analgesics.

Cough suppression is an important clinical action of opiates. Whereas analgesic effects of opiates are stereospecific with virtually all activity residing usually in the (−)-isomer, (+)-opiates often have significant cough-suppressive activity. Dextromethorphan, a (+)-isomer, is the most widely used cough-suppressant in the United States. Receptor binding sites have been identified for cough-suppressant agents such as [^{3}H]dextromethorphan and (−)-[^{3}H]codeine (*48*). The [^{3}H]dextromethorphan sites are localized to brainstem areas (as revealed by autoradiography), such as the floor of the fourth ventricle, known to be involved in cough reflexes (*49*).

Autoradiographic visualization of opiate receptors has contributed greatly to our understanding of how these drugs influence body functions. Neuropharmacologists have debated the site of analgesic action of opiates, some maintaining that all opiate analgesia occurs in the brain, while others had evidence for analgesic actions at a spinal cord level. Autoradiographic studies show a dense band of opiate receptors localized in the substantia gelatinosa, a narrow zone in the dorsal part of the spinal cord where sensory neurons enter the cord (*29, 50*). The substantia gelatinosa contains many small interneurons and is the first way station for integrating incoming sensory information. The opiate receptors in the substantia gelatinosa may account for opiate analgesia observed with direct injections of morphine into the spinal cord area. Electrical stimulation in the periaqueductal gray of the brainstem elicits analgesia in animals and in humans which can be blocked by the opiate antagonist naloxone (*51*). Injections of morphine in this area of the brain are also analgesic (*52*). An intense cluster of opiate receptors occurs in the periaqueductal gray. These, along with other localizations of opiate receptors in the brain, provide an explanation for both

supraspinal and spinal cord levels of opiate analgesia.

Emotional regulation in the brain is subserved by a group of structures called the limbic system. Opiate receptors are highly concentrated in numerous structures of the limbic system, especially the amygdala. The neurotransmitter norepinephrine is thought to be involved in affective states. Many of the norepinephrine neurons in the brain have their cells in a small nucleus in the brainstem, the locus coeruleus. The locus coeruleus possesses one of the most dense clusters of opiate receptors in the entire brain.

Bird and Kuhar (*53*) utilized the locus coeruleus to demonstrate that opiate binding sites in the brain are functional opiate receptors. They made electrical recordings at single cells in the locus coeruleus and found that low concentrations of opiates inhibited cell firing, an effect that was blocked by opiate antagonists. When the recording electrode was moved less than a millimeter outside the locus coeruleus, opiates no longer influenced firing.

Results of autoradiographic studies have revealed other effects of opiates. Opiates constrict the pupils of the eye so well that the police routinely identify heroin addicts by their "pinpoint" pupils. High densities of opiate receptors in

Table 1. Drug and neurotransmitter receptor types and subtypes.

Subtype	Properties
	Opiate type
μ	Morphine-selective; localized in pain modulating brain regions.
μ_1	Identified by very high affinity binding of numerous opiates; blocked selectively by naloxonazine; meptazinol a specific agonist; implicated in analgesia but not respiratory depression.
δ	Enkephalin-selective; localized in limbic brain regions.
κ	Mediates sedating, less addicting analgesia; localized to deep layers of cerebral cortex; dynorphin has high affinity; mediates rabbit vas deferens contractions.
σ	Naloxone insensitive; mediates psychotomimetic opiate effects; concentrated in hippocampus.
ϵ	β-Endorphin selective; mediates rat vas deferens contractions.
Cough-suppressant	Dextromethorphan-selective; reversed stereospecificity; localized to fourth ventricle floor which regulates cough reflexes.
	Calcium antagonist type
Dihydropyridine	Binding dependent on Ca^{2+} and blocked by ionic calcium antagonists; regulated allosterically by verapamil; localized to molecular layer dentate gyrus, external plexiform layer olfactory bulb.
Verapamil	Inhibited by physiologic Ca^{2+} levels; linked to behavioral activation by diphenylbutylpiperidine neuroleptics, sexual and cardiac effects of the phenothiazine thioridazine, and antidiarrheal actions of loperamide and diphenoxalate.
Diltiazem	Can involve same site as verapamil in part; allosterically regulated by dihydropyridines.
	Adenosine type
A_1	Labeled by [^{3}H]cyclohexyladenosine, [^{3}H]phenylisopropyladenosine, 2-[^{3}H]chloroadenosine; lowers adenylate cyclase; adenosine analogs potent at nanomolar concentrations; stereospecific for phenylisopropyladenosine; localized to molecular layers of hippocampus and cerebellum, medial geniculate; contained on nerve terminals of cerebellar granule cells and retinal ganglion cell projections to superior colliculus.
A_2	Stimulates adenylate cyclase; adenosine analogs potent at micromolar concentrations; little stereoselectivity for phenylisopropyladenosine; labeled with 5′-*N*-[^{3}H]ethylcarboxamide adenosine.
	GABA type
A	Muscimol-selective; postsynaptic to GABA neurons; inhibited by calcium; antagonized by convulsant bicuculline.
B	Baclofen-selective; on GABA and other nerve terminals; stimulated by calcium; bicuculline-resistant.
Sedative-convulsant	Labeled by convulsants [^{3}H]dihydropicrotoxinin and [^{35}S]-*t*-butylbicyclophosphorothionate; regulated by chloride and barbiturates; linked to benzodiazepine and $GABA_A$ receptors.
	Dopamine type
D_1	Enhances adenylate cyclase; labeled by [^{3}H]thioxanthenes; absent in pituitary; present in parathyroid.
D_2	Lowers adenylate cyclase; labeled by [^{3}H]butyrophenones; present in anterior pituitary; responsible for antipsychotic and extrapyramidal actions.
	Serotonin (5-HT) type
5-HT_1	Labeled with [^{3}H]5-HT, which is potent at nanomolar concentrations; classical 5-HT antagonists are weak; regulated by guanine nucleotides, possibly linked to adenylate cyclase; mediates contraction of dog basilar artery.
5-HT_2	Labeled with [^{3}H]spiperone and [^{3}H]ketanserin; micromolar 5-HT; less affected by guanine nucleotides; mediates behavioral "5-HT syndrome," contraction of estrous rat uterus, dog and rabbit femoral and rat caudal arteries, and rat and rabbit aorta and jugular vein.
	α-Adrenergic type
α_1	Postsynaptic in sympathetic system; prazosin- and indoramin-selective; acts through Ca^{2+} channels; little affected by guanine nucleotides.
α_2	Located on sympathetic nerve terminals to regulate norepinephrine release but also postsynaptic, especially in brain; clonidine selective agonist; yohimbine- and piperoxan-selective antagonists; lowers adenylate cyclase.
	β-Adrenergic type
β_1	Epinephrine and norepinephrine equally potent agonists; practalol-selective antagonist; more in heart than lung; regional variations in brain; neuronal localization.
β_2	Epinephrine more potent than norepinephrine; terbutaline- and salbutamol-selective agonists; more in lung than heart; few regional variations in brain; more on glia than neurons.
	Muscarinic cholinergic type
M_1	Concentrated in sympathetic ganglia, corpus striatum, and stomach; pirenzipine-selective antagonist; closes K^+ channels.
M_2	Concentrated in hindbrain, cerebellum, and heart; regulated by gallamine and GTP; inhibits adenylate cyclase.

the pretectal nuclei of the brainstem, which are involved in regulating pupillary diameter, may explain these effects. Opiate overdoses kill by depressing respiration, and this may be related to opiate receptor concentrations in vagal nuclei of the brainstem, which participate in respiratory reflexes.

Differential functions of opiate receptor subtypes are suggested by selective receptor localizations in the brain. In autoradiographic investigations, my colleagues and I found μ receptors were most enriched in brain regions involved in integrating information about pain perception and that δ receptors were more highly concentrated in limbic structures (*54*). This suggests that μ receptors may be responsible in large part for analgesic effects of morphine, whereas δ receptors may mediate euphoric effects. The κ receptors were localized to the deep layers of the cerebral cortex (*55*). Cells in these layers project to the thalamus to modulate sensory input from the thalamus to the cerebral cortex. It is possible that these κ receptors participate in the effects of κ opiates on levels of consciousness.

Strong evidence that enkephalins are the major endogenous opiate neurotransmitters of the brain stems from autoradiographic localizations of opiate receptors. Immunohistochemical studies with antiserum to the enkephalins permitted a visualization of enkephalin-containing neurons (*56*). The resultant localization of enkephalin neuronal systems corresponds closely to the localization of opiate receptors. By contrast, the distribution of other opiate peptides such as β-endorphin differs from most opiate receptors (*57*).

Calcium Antagonist Receptors

Calcium ions regulate numerous cellular events. In the brain, calcium is required for neurotransmitter release and plays a role in the actions of some neurotransmitters on cells. Biophysicists have identified several different types of calcium channels in cell membranes. One of these, a voltage-dependent calcium channel, also referred to as a "slow" calcium channel, is of considerable importance in the contractions of smooth muscle and the rhythm-generating mechanisms of the heart. Several drugs impair the movement of calcium through the voltage-dependent channels and are referred to as organic calcium antagonists (*58*). These drugs are used to treat hypertension, angina, cardiac arrhythmias, and migraine headaches. Several structural classes of calcium antagonists have been differentiated, including dihydropyridines such as nifedipine, verapamil-like drugs, and diltiazem.

Receptor binding studies have clarified the way in which these drugs act. Up to now it has been possible to label binding sites most reliably with dihydropyridines such as [^{3}H]nitrendipine (*59, 60*). Direct comparison of the potencies of numerous dihydropyridines in blocking calcium-induced contractions of smooth muscle and in binding to the same muscle tissue has established that the labeled sites are pharmacologically relevant (*60*). The relation of the receptor sites to calcium channels was clarified by the observation that [^{3}H]nitrendipine binding is abolished in the absence of calcium but can be restored by physiological concentrations of calcium or other divalent cations that mimic the actions (*61*).

Some cations, such as lanthanum and cobalt, are potent inorganic calcium antagonists. Others, such as manganese, mimic the physiologic actions of calcium at low concentrations but block calcium at higher concentrations. Cation effects on [^{3}H]nitrendipine binding help explain the agonist and antagonist actions of cations (*61*). The stimulation of [^{3}H]nitrendipine binding by calcium is blocked by low concentrations of cobalt and lanthanum. Manganese exerts a biphasic effect on [^{3}H]nitrendipine binding, stimulating binding at lower concentrations and inhibiting it at higher concentrations. Manganese blocks the stimulatory effects of calcium.

Pharmacologic studies have indicated that drugs of the verapamil and diltiazem class act at sites different from those of the dihydropyridines. This is consistent with the failure of these drugs to compete directly for [^{3}H]nitrendipine binding. However, Yamamura and associates showed that verapamil can influence [^{3}H]nitrendipine binding via a distinct site which is allosterically linked to the dihydropyridine receptor (*62*). Whereas verapamil inhibits [^{3}H]nitrendipine binding, diltiazem stimulates it. It was not clear whether verapamil and diltiazem act at the same or at different sites. Moreover, sites of action were unclear for other calcium antagonists, such as prenylamine and lidoflazine. Verapamil, diltiazem, prenylamine, lidoflazine, and several other related agents share a common site of action distinct from but allosterically related to the dihydropyridine receptor (*63*). At this allosteric site, these drugs increase or decrease the affinity of [^{3}H]nitrendipine. Verapamil maximally decreases the affinity of [^{3}H]nitrendipine 2.4-fold whereas prenylamine can decrease its affinity tenfold. Accordingly, verapamil can maximally inhibit [^{3}H]nitrendipine binding only 30 to 40 percent, but prenylamine can reduce binding to negligible levels. Since a drug such as verapamil can reverse the inhibition of binding by a drug such as prenylamine, these two agents must act at the same site. Drugs such as verapamil reduce the affinity of [^{3}H]nitrendipine binding by accelerating its dissociation from receptors, whereas diltiazem enhances binding by slowing its dissociation rate (*63*). Additional insight into the nature of subtypes of calcium antagonist receptors comes from new methods for identifying receptor binding sites for [^{3}H]verapamil (*64*) and [^{3}H]diltiazem (*65*), compounds that appear to reflect distinct sites: [^{3}H]diltiazem binding is regulated allosterically by dihydropyridines as is [^{3}H]verapamil binding in muscle transverse tubules.

The diversity of structures associated with calcium antagonist properties prompted a screening of numerous drugs for possible calcium antagonist effects. Evaluating a large number of antischizophrenic neuroleptic drugs indicated that agents of the diphenylbutylpiperidine class, such as pimozide, clopimozide, fluspirilene, and penfluoridol, are quite potent in influencing [^{3}H]nitrendipine binding, acting allosterically at the verapamil site (*66*). The diphenylbutylpiperidines have similar potencies in blocking effects of calcium in smooth muscle as in competing at receptor binding sites. Antischizophrenic neuroleptic drugs are thought to exert their therapeutic actions by blocking dopamine receptors (*67*). The diphenylbutylpiperidines are almost as potent calcium antagonists as they are dopamine receptor blockers (*66*). Thus, at therapeutic doses, calcium channels in the brain should be influenced by these drugs. Other neuroleptics, including the butyrophenones and phenothiazines, are weaker calcium antagonists. Diphenylbutylpiperidines, butyrophenones, and phenothiazine neuroleptics all display similar antipsychotic activities and elicit the well-known extrapyramidal side effects attributed to dopamine receptor blockade. We reasoned that behavioral effects caused by diphenylbutylpiperidines but not by the other types of neuroleptics might provide a clue to the function of voltage-dependent calcium channels in the brain. A review of clinical data, including those from several coded (double-blind) control studies, indicated specific differences between therapeutic effects of diphenylbutylpiperidines and other neu-

roleptics (*68*). These properties were evident for all four of the diphenylbutylpiperidines in clinical use.

Traditional phenothiazine and butyrophenone neuroleptics are highly effective in relieving the "positive" symptoms of schizophrenia, such as florid hallucinations and delusions. The "negative" symptoms of schizophrenia, such as emotional withdrawal and poverty of speech and affect, are not as responsive to these agents. The diphenylbutylpiperidines consistently ameliorate both the negative and positive symptoms of schizophrenia (*68*). They seem to have an activating effect and reproducibly decrease the emotional withdrawal of patients. A directed search for drugs that are centrally active calcium antagonists as well as dopamine blockers might provide other agents with similar therapeutic benefits. Centrally active calcium antagonists lacking dopamine blocking properties might also exert therapeutic behavioral effects.

The highest density of [^{3}H]nitrendipine binding sites occurs in the brain, but functional roles of voltage-dependent calcium channels are established most strongly in blood vessels and the heart. Further, calcium-dependent functions of neurons, such as neurotransmitter release, are not influenced to any degree by calcium antagonist drugs. What might be the function of the high density of these receptors in the brain?

The well-known association of voltage-dependent calcium channels with blood vessels suggested that this might be the site of receptors in the brain. However, our autoradiographic studies of [^{3}H]nitrendipine binding in the brain revealed a very different pattern (*69*). Receptor sites are highly localized to synaptic zones with very little binding at blood vessels. There are considerable regional variations. Highest densities occur in the limbic system, especially the hippocampus. For instance, a dense band of receptors lies in the molecular layer of the dentate gyrus, but adjacent granule cells in this part of the brain are devoid of receptors. Within the olfactory bulb, receptors are highly concentrated in the external plexiform layer whereas the adjacent glomerular layer has far fewer grains.

This pattern resembles the distribution of many neurotransmitter receptors. Thus, like opiate receptors, these sites may therefore be receptors for some normally occurring neurotransmitter that exerts its synaptic effects by altering calcium passage through membranes.

Some peripheral side effects of neuroleptics may reflect calcium antagonist properties. The phenothiazine neuroleptics vary in their side effects. For example, thioridazine inhibits ejaculation in a large number of patients even in the presence of normal erections, a side effect that occurs infrequently with most phenothiazines or butyrophenones. Phenothiazines can elicit cardiac arrhythmias, due in part to the local anesthetic actions of the drugs; thioridazine has a greater propensity to elicit these effects than other neuroleptics. At concentrations corresponding to therapeutic blood levels, thioridazine has substantial calcium antagonist activity whether monitored by receptor binding or by the blockade of calcium-induced smooth muscle contraction (*70*). At therapeutic blood levels, other phenothiazines are much less active. The smooth muscle system used to monitor calcium antagonist effects with thioridazine was the vas deferens, whose contractions account for ejaculation. Inhibition of ejaculation by thioridazine therefore appears related to the calcium antagonist properties of the drug. The electrocardiographic abnormalities occurring with thioridazine treatment resemble, in part, arrhythmias that can occur with verapamil, suggesting that the relatively high incidence of cardiac arrhythmias with thioridazine may also be related to calcium antagonism. While the high therapeutic blood levels of thioridazine display calcium antagonist activity, thioridazine is considerably less potent as a calcium antagonist than as a dopamine blocker, so that one would not anticipate substantial calcium channel blockage in the brain with therapeutic doses of thioridazine.

Drugs thought to act by other mechanisms may also work through calcium channels. The major antidiarrheal drug loperamide is a potent opiate agonist. However, its antidiarrheal effects are only partially reversed by the opiate antagonist naloxone. Loperamide has calcium antagonist actions both in receptor binding and smooth muscle contraction paradigms in therapeutically relevant concentrations (*71*). Thus, a major part of the antidiarrheal effects of loperamide stems from calcium antagonism, which is consistent with the well-known constipating effects of verapamil.

Adenosine Receptors

Adenosine has many functions in the body. It is the precursor of adenosine triphosphate (ATP), an energy source, a building block of nucleic acids, and a potent vasodilator. Further, it inhibits platelet aggregation and constricts the bronchial tree. In the brain adenosine inhibits neuronal firing (*72*), and recent evidence indicates that a major part of brain adenosine is contained in neurons. Whether adenosine serves as a neurotransmitter in the brain is not clear.

Adenosine influences adenylate cyclase via at least two apparently distinct receptor subtypes. At A_2 receptors adenosine stimulates adenylate cyclase, while at A_1 receptors it inhibits adenylate cyclase (*73*). Xanthines such as theophylline or caffeine block both A_1 and A_2 effects on cyclic AMP.

Because of its numerous actions throughout the body, we sought to label adenosine receptors by binding techniques. In contrast to the situation with other receptors, neither adenosine nor known xanthine adenosine antagonists could serve as suitable ligands. To block adenosine effects requires micromolar concentrations of xanthines, indicating inadequate affinity to permit receptor labeling. Adenosine itself is metabolized very rapidly in tissue membranes and so cannot serve as a binding ligand.

Accordingly, new ligands were synthesized. Attaching a cyclohexyl group to the amine moiety of adenosine prevented enzymatic degradation, and the resultant ligand [^{3}H]cyclohexyladenosine ([^{3}H]CHA) proved to have affinity (in nanomolar quantities) for adenosine receptors (*74*). Varying substituents on the xanthine molecule provided 1,3-diethyl-8-phenylxanthine (DPX) which is more potent in blocking adenosine effects than theophylline or caffeine and provided a suitable antagonist ligand (*74*). Other investigators labeled adenosine receptors with [^{3}H]phenylisopropyladenosine ([^{3}H]PIA) (*75*) or 2-[^{3}H]chloroadenosine (*76*). The drug specificity of binding sites labeled by these ligands reflected A_1 receptors. *N*-Ethylcarboxamidoadenosine (NECA) has some A_2 receptor preference in its effects on adenylate cyclase, and [^{3}H]NECA binding appears to involve A_2 as well as A_1 receptors (*77*).

There may exist receptor heterogeneity beyond the A_1 and A_2 dichotomy. For instance, in evaluating [^{3}H]CHA binding to receptors in the brains of different species we found marked differences in drug effects. Thus, xanthines such as DPX vary as much as 500-fold in potency at adenosine A_1 receptor binding sites in various species (*78*).

In early studies of cyclic AMP by Sutherland and Rall, caffeine was shown to inhibit the cyclic AMP–destroying enzyme phosphodiesterase (*79*). It was generally assumed that caffeine exerts its stimulant effects by inhibiting phospho-

diesterase, thereby permitting an accumulation of cyclic AMP in the brain. However, inhibitors of this enzyme that are more potent than caffeine lack central stimulant effects. My colleagues and I wondered whether the known ability of caffeine to block adenosine receptors (*80*) might be responsible for its behavioral effects. Since we could measure adenosine receptors in binding studies, we evaluated the relative potencies of xanthines as adenosine antagonists and in parallel studies examined their behavioral influences in rodents (*81*).

In general, potencies in blocking adenosine receptors correlated with stimulation of locomotor activity in mice. Moreover, the concentrations of caffeine that block adenosine receptors corresponded to those in the brains of humans after their drinking a few cups of coffee. However, some xanthines that were effective adenosine antagonists did not exhibit locomotor stimulant activity. Moreover, xanthines such as caffeine showed a biphasic effect, depressing locomotor activity at low doses and enhancing it at higher doses. To focus selectively on the behavioral aspects of xanthine interactions with adenosine systems, we treated mice with the potent adenosine agonist PIA. Low doses of PIA depressed locomotor activity. Not only did xanthines block this effect, but they transformed it into a marked stimulation of locomotor activity, greater than that observed with the xanthines alone. Xanthines that had been ineffective in enhancing locomotor activity when administered alone but that were potent adenosine antagonists were highly active in reversing the PIA-induced depression of activity (*81*). Low doses of caffeine, which by themselves elicit locomotor depression, produced locomotor stimulation when combined with a depressant dose of PIA.

The mechanism for the paradoxical reversal by xanthines of PIA locomotor depression became apparent in more extensive studies (*82*). When still lower doses of PIA were administered to mice, locomotor enhancement was observed. Thus, at a very high affinity site PIA causes locomotor stimulation while at a site of somewhat lesser affinity it elicits depression. Apparently caffeine blocks the effects of PIA at sites that produce locomotor depression, thus unmasking the locomotor stimulatory potential of PIA.

If behavioral effects in rodents are similar to those in humans, this body of work indicates that the behavioral stimulant actions of caffeine involve adenosine receptor blockade. Whether other xanthine effects, such as the bronchodilatory therapeutic actions of theophylline, involve adenosine is not clear. To investigate such questions directly, it would be desirable to measure adenosine receptor binding in peripheral tissues. Thus far extracerebral adenosine receptor binding has been reported only in fat cells (*83*) and in the testes (*76, 84*).

Adenosine receptors have been visualized by autoradiography with [^{3}H]CHA (*85, 86*) to determine the site of adenosine action in the brain. Highest densities occur in the molecular layer of the cerebellum, the molecular and polymorphic layers of the hippocampus and dentate gyrus, the medial geniculate body, certain subthalamic nuclei, and the lateral septum. Adenosine inhibits neuronal activity largely by presynaptic mechanisms, presumably involving inhibition of the release of excitatory neurotransmitters (*72, 87*). My colleagues and I wondered how the observed receptor localizations account for the inhibitory actions of adenosine brain.

The cerebellum was chosen for exploration of the cellular localization of adenosine receptors because it has five well-characterized cell types. The Purkinje, Golgi II, stellate, and basket cells are inhibitory. The only excitatory neuron in the cerebellum is the granule cell, whose axons give rise to parallel fibers in the molecular layer of the cerebellum that synapse on Purkinje cells. We evaluated the autoradiographic pattern of adenosine receptors in the cerebellum of various mutant mice (*88*). Weaver mice, in which the granule cells are selectively lost, also lack adenosine receptors in the molecular layer. In Reeler mice, whose granule cells occur in an aberrant layer, adenosine receptors display a similarly aberrant localization. Thus, in the cerebellum the adenosine receptors appear associated with axons and terminals of the major excitatory neurons.

The neurotransmitter of the optic nerve, which passes from the eye to the superior colliculus and the lateral geniculate, is excitatory. Degeneration of the optic nerve was elicited by unilateral enucleation of the eye in rats. Adenosine receptors were depleted in the contralateral superior colliculus (*88*). Thus, within the superior colliculus adenosine receptors are associated with excitatory axons and terminals. In other parts of the brain, lesions of excitatory fibers did not deplete adenosine receptors. In some but not all areas, therefore, one can account for the synaptic inhibitory actions of adenosine on the basis of a selective localization of adenosine receptors to terminals of excitatory neurons.

GABA and Benzodiazepine Receptors

GABA is the major inhibitory neurotransmitter in the brain. The GABA receptors were first labeled by the direct binding of [^{3}H]GABA (*8*). In pharmacologic studies the antianxiety benzodiazepine drugs seem to act by facilitating synaptic actions of GABA. However, no direct effects of benzodiazepines on [^{3}H]GABA binding were apparent in initial investigations. Further, when specific benzodiazepine receptor binding sites could be identified with [^{3}H]diazepam, GABA did not appear to compete for these sites (*89*). This prompted a search for "the endogenous Valium," in analogy with the successful identification of the enkephalins as the endogenous morphine-like factors. Several investigators then showed that low concentrations of GABA and related amino acids can enhance benzodiazepine binding in proportion to their GABA-like synaptic effects (*90*). GABA does not act at the same receptor binding site as the benzodiazepines but at a presumably allosterically linked site. This interaction between the benzodiazepine and GABA binding sites is analogous to the allosteric interaction of the distinct calcium antagonist receptors described above. The benzodiazepine and associated GABA recognition sites are closely linked, since solubilization and extensive purification of receptors fail to dissociate the GABA receptor and benzodiazepine binding (*91*). Besides the distinct recognition sites for GABA and benzodiazepines, barbiturates modulate benzodiazepine binding through yet another site, a "sedative-convulsant" receptor, which accounts at least in part for their pharmacologic effects (*92*). The sedative-convulsant receptor can be labeled with convulsants such as [^{3}H]dihydropicrotoxinin and [^{35}S]*t*-butylcyclophosphorothionate (*92*). Chloride ions influence benzodiazepine binding, which might indicate some linkage with the chloride ion channel where GABA elicits synaptic hyperpolarization (*93*).

Besides classical postsynaptic GABA receptors, termed $GABA_A$, receptors on nerve terminals of GABA and other transmitter-containing neurons can be identified with [^{3}H]GABA or the antispasticity drug [^{3}H]baclofen; these have been designated $GABA_B$ receptors (*94*). The $GABA_B$ receptors regulate neurotransmitter release, are stimulated by calcium, and are unaffected by the classical GABA antagonist bicuculline.

Benzodiazepine receptors illustrate the value of receptor binding techniques in drug discovery. Numerous nonbenzo-

diazepine structures compete potently at benzodiazepine receptors and may provide useful antianxiety agents whose novel structures may convey novel therapeutic actions (*95*). The identification of chemicals that bind potently to benzodiazepine receptors but are devoid of anxiolytic effects has led to the classification of selective benzodiazepine antagonists (*96*). Several types of benzodiazepine antagonists and "inverse agonists" have been described, some of which elicit extreme anxiety, almost a panic state, in humans (*97*).

Benzodiazepine receptors also appear to exist as at least two subtypes. The first evidence came with the observation that the triazolopyridazine CL218872 was more potent in competing for benzodiazepine binding in the cerebellum than in the hippocampus (*98*). It was proposed that the cerebellum is enriched in a receptor subtype, designated type I, with selectively high affinity for CL218872, while the hippocampus primarily possesses type II receptors which have less affinity for the drug. The existence of distinct type I and type II receptor proteins was brought into question by discoveries that the differential effects of CL218872 in the two brain regions varied as a function of temperature (*99*). Perhaps the apparent receptor subtypes represent varying, temperature-sensitive conformations of a single protein. However, other evidence favors distinct type I and type II benzodiazepine receptor proteins. In one region of the brain, the substantia nigra, lesions of the descending pathway from the caudate nucleus, which contains GABA neurons, deplete apparent type II receptors while augmenting the numbers of type I receptors (*100*). Thus, the type II receptors appear to be localized on nerve endings of the descending pathway, while the type I receptors are postsynaptic. This fits with subcellular fractionation studies indicating that type I receptors are associated with postsynaptic densities (*101*).

Distinct electrophoretic bands apparently reflecting types I and II benzodiazepine receptors have been found by some investigators (*102*), although others find only single bands (*103*). Apparent type I and type II receptors have been separated physically by detergent treatment (*104*). Detergents such as Triton X-100 selectively solubilize receptors with a type II drug profile. Solubilization of apparent type I receptors requires a combination of detergent and high salt concentrations, which fits with the solubilization properties of postsynaptic densities. Types I and II receptors may differ in their relation to chloride ion channels in that solubilized type II but not type I receptors are regulated by chloride ions (*105*).

The discrimination of type I from type II benzodiazepine receptors provides a strategy for developing selective drugs. If one of these receptors mediates anxiolytic effects of benzodiazepines while the other accounts for sedative actions, drugs selective for receptor subtypes might permit therapeutic advances. The relative behavioral roles of these receptor subtypes is presently unclear (*98, 106*).

Cyclopyrrolone drugs such as suriclone and zopiclone, whose therapeutic effects are indistinguishable from benzodiazepines, act through yet another receptor which is linked to benzodiazepine receptors (*107*). The cyclopyrrolone receptor is associated more closely with the sedative-convulsant receptor, since cyclopyrrolones are hundreds of times more potent than benzodiazepines in regulating sedative-convulsant receptors.

Biogenic Amine Receptors

Dopamine receptors also illustrate several principles. At least two subtypes of dopamine receptors can be distinguished: D_1 receptors, which are associated with enhancement of adenylate cyclase and are labeled selectively by phenothiazines such as [^{3}H]fluphenazine or the related agent [^{3}H]flupentixol (*108*), and D_2 receptors, which are related to reduction of adenylate cyclase activity (*109*) and are labeled selectively by butyrophenones such as [^{3}H]haloperidol and [^{3}H]spiperone (*10*). The antipsychotic actions of neuroleptics correlate closely with the relative affinities of drugs for D_2 but not D_1 receptors (*67*). The pituitary gland has dopamine receptors that mediate prolactin release, and the dopamine agonist ergot bromocryptine relieves acromegaly and the amenorrhea and impotence associated with hypersecretion of prolactin. These effects also involve D_2 receptors for which bromocryptine has high affinity, while it lacks agonist effects at D_1 receptors. Even the extrapyramidal side effects of neuroleptics are attributable to blockade of D_2 rather than D_1 receptors. Thus, dopamine receptors account for major clinical actions of drugs. Except for some influences on parathyroid hormone release (*110*), the D_1 receptor function remains to be characterized. Additional postulated D_3 and D_4 receptor binding sites appear to be simply high affinity forms of D_1 or D_2 receptors (*111*).

Numbers of dopamine receptors vary with transmitter exposure. Unilateral destruction of the dopamine neuronal input to the corpus striatum provokes a marked increase in numbers of dopamine receptors, presumably reflecting an attempt to compensate for the dopamine deficiency (*112*). Similarly, chronic treatment with neuroleptics that block dopamine receptors augments receptor number (*112*). The dopamine receptor increases following denervation or neuroleptic treatment are associated with behavioral supersensitivity to dopamine-mimicking drugs (*113*). Patients treated over a long period with neuroleptics develop motor manifestations suggesting dopamine receptor supersensitivity. These symptoms, referred to as tardive dyskinesia, are sometimes not reversible and represent one of the most serious side effects of neuroleptic drug treatment. Tardive dyskinesia may be related to dopamine D_2 receptor augmentation after prolonged blockade of receptors by high doses of neuroleptics.

Serotonin receptors can be readily labeled in binding studies, and at least two distinct subtypes have been identified: 5-HT_1 receptors, which are labeled by [^{3}H]serotonin, and 5-HT_2 receptors, which can be labeled with [^{3}H]spiperone, a neuroleptic equally potent in blocking serotonin and dopamine receptors (*25*). Certain behavioral effects of serotonin are associated primarily with 5-HT_2 sites, while some influences of serotonin-related agents on adenylate cyclase may reflect 5-HT_1 receptors. The 5-HT_1 and 5-HT_2 receptors also differentially regulate contractions of various blood vessels (*25*).

Other biogenic amine receptors that can be labeled by ligand binding in the brain include α- (*11*) and β- (*12*) adrenergic receptors as well as histamine H_1 receptors (*114*). Both α and β receptors exist in subtypes which are differentially localized and have clinical implications (*115*). The drug specificity of histamine H_1 receptor binding sites varies in different species (*116*) and organs (*117*), suggesting receptor heterogeneity. ^{3}H-Labeled ligand binding to histamine H_2 receptors in brain tissue has been reported (*118*). Muscarinic (*7*) acetylcholine receptors can also be examined by binding techniques in the brain and appear to reflect subtypes, each of which can occur in different conformations (*119*). Drugs with selectivity for M_1 and M_2 muscarinic cholinergic receptors localized differentially in various organs have specific therapeutic utility. Thus, the gastrointestinal selectivity of the M_1 antagonist pirenzipine facilitates the thera-

py of peptic ulcer. Receptor sites on brain membranes for numerous neuropeptides have been labeled, including thyrotropin-releasing hormone (*120*), vasoactive intestinal polypeptide (*121*), neurotensin (*122*), substance P (*123*), cholecystokinin (*124*), bradykinin (*125*), somatostatin (*126*), angiotensin (*127*), bombesin (*128*), and insulin (*129*).

The ligand binding strategy has been employed to study other neurotransmitter-associated sites in brain membranes. Membrane-bound enzymes can be labeled with potent inhibitors such as [^{3}H]harmaline for monoamine oxidase (*130*) and [^{3}H]captopril for angiotensin-converting enzyme (*131*).

Sites related to amine uptake inactivation have been labeled with [^{3}H]imipramine for serotonin (*132*), [^{3}H]desipramine for norepinephrine (*133*), and [^{3}H]mazindol for dopamine uptake sites (*134*). Variations in numbers of norepinephrine uptake recognition sites with chronic drug treatment suggest that altered levels of transmitter regulate transmitter inactivation by the uptake (*135*).

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