Receptors for the Age of Anxiety: Pharmacology of the Benzodiazepines

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Your worst enemy, he reflected, was your own nervous system. At any moment the tension inside you was liable to translate itself into some visible symptom. . . . And what was frightening was that the action was quite possibly unconscious.

—GEORGE ORWELL, 1984

Throughout history, anxiety has been recognized as an inherent part of man's being. Discussion of the origins of anxiety has become explicit in the 20th century and is a frequent theme in today's literature. The definition of anxiety is as varied as the experience itself, and its biological basis is obscure. While anxiety may be thought of as an unpleasant state, characterized by uneasiness and apprehension (*1*), it is also a strong motivating force in many forms of behavior and, like fear, has fundamental adaptive and perhaps evolutionary significance. Pathological or excessive anxiety is clearly undesirable, and its postulated relation to many forms of "purely" physical disease make understanding its origins and treatment a major concern (*1*). In recent years, the "minor tranquilizers" have become increasingly popular for the treatment of anxiety, and the benzodiazepines are the most widely prescribed minor tranquilizers in current use. Recent advances in our understanding of how benzodiazepines produce their therapeutic effects have also provided clues to the basic neurochemical mechanisms in the central control of anxiety, seizures, muscle relaxation, and perhaps even sleep.

History and Use

In 1960 chlordiazepoxide (Librium) became the first 1,4-benzodiazepine (*2*) introduced into clinical use. It was rapidly followed by a more potent analog, diazepam (Valium), and in 1970 by flurazepam (Dalmane). Chlordiazepoxide and diazepam continue to be used widely in clinical practice as muscle relaxants, anticonvulsants, anxiolytics, and hypnotics (sleeping pills). In 1977, 54 million prescriptions were written for diazepam and 13 million for chlordiazepoxide (*3*). Flurazepam is specifically marketed in North America as a hypnotic (nitrazepam is used for this purpose in several European countries) and in 1977 accounted for 53 percent of all hypnotics prescribed (13.6 million prescriptions) (*3*). A conservative estimate (*4*) indicates that at least 8000 tons of the benzodiazepines were consumed in the United States in 1977.

When administered orally, the benzodiazepines become widely distributed in the body and, because of their chemical structure, they accumulate preferentially in lipid-rich tissues such as adipose and brain. They are primarily metabolized by the liver and excreted by the kidney. Pharmacologically active metabolites of the benzodiazepines are frequently long-lived, and their delayed excretion may account for impaired "next-day" functioning after their use as hypnotics (*3*). Cumulative dose effects after long-term use has also been reported (*3*). Although physical addiction to benzodiazepines is not as significant a problem as with other psychopharmacological agents, psychological dependence on benzodiazepines has been reported and physical withdrawal symptoms have been observed after abrupt cessation of moderate to high doses (*5*). Interactions with other central nervous system (CNS) depressants, particularly alcohol, are a major problem in clinical practice. Nevertheless, a wide margin of safety between the therapeutic and toxic doses of benzodiazepines has been consistently observed (*5*).

Behavioral Effects in Animals and Man

The benzodiazepines are used to modify behavior in a wide variety of clinical situations ranging from the treatment of anxiety and insomnia to the management of alcohol withdrawal (*5*). Their established efficacy in a number of seemingly diverse neuropsychiatric conditions stems from at least four distinct behavioral effects. In man these include: anticonvulsant, muscle relaxant, anxiolytic, and sedative-hypnotic properties. Although it has been suggested that the antianxiety effects of the benzodiazepines may result from a combination of the drug's muscle relaxant and sedative effects, each of these effects alone is insufficient for anxiolytic activity. Therefore, it is more likely that the various behavioral effects of the benzodiazepines have a closely related neuropharmacological mechanism or may reflect degrees of action along a single continuum of effects. These hypotheses are supported by the high degree of predictive correlation among the various behavioral tests for benzodiazepine activity and the positive correlations observed between the animal tests and anxiolytic activity in man (*6*).

Most of the clinically used anxiolytics (benzodiazepines and meprobamate, for example) display some degree of anticonvulsant activity in both laboratory animals (*7*) and man (*8*). However, the benzodiazepines seem to be selectively effective in preventing pentylenetetrazol (Metrazol)-induced seizures. Furthermore, the relative potencies of various benzodiazepines in protecting mice against pentylenetetrazol-induced seizures correlate well with their relative clinical potencies as anxiolytics. Since measuring seizure activity is a relatively simple procedure, prevention of pentylenetetrazol-induced seizures in mice is the most commonly used initial screening test for potential anxiolytic drugs (*9*). Many investigators maintain that humans do not develop overt tolerance to the anxiolytic effects of the benzodiazepines (*10*) although this question is controversial (*11*). Animals do not develop tolerance to the antipentylenetetrazol activity of the benzodiazepines (*12*). This is in contrast to the effects of these drugs in preventing seizures induced by both strychnine and bicuculline, where tolerance develops in animals subjected to long-term treatment with benzodiazepines (*12*). These studies suggest

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that an understanding of the biochemical mechanisms in either the production or inhibition of pentylenetetrazol-induced seizures may lead to an understanding of the anxiolytic action of the benzodiazepines.

The ability of the benzodiazepines to produce muscle relaxation in laboratory animals was observed in the initial behavioral studies of these agents (*13*). Although muscle relaxant activity is not unique to the benzodiazepines (all commonly used anxiolytics share this property), the extreme potency of the benzodiazepines as muscle relaxants (*6*) makes this a selective effect. It is generally agreed that the muscle relaxation produced by the benzodiazepines is centrally rather than peripherally mediated (*14*). The relatively high concentrations of benzodiazepine necessary to inhibit neuromuscular impulse conduction in isolated nerve-and-muscle preparations (*15*) make a solely peripheral action highly unlikely. Although significant neurophysiological effects have been demonstrated at both spinal and supraspinal levels, the exact CNS sites responsible for the muscle relaxant properties of the benzodiazepines are unknown. Also, there seems to be a close relation between their effective doses as muscle relaxants in animals and their anxiolytic doses in man (*6*).

One of the more impressive behavioral effects of the benzodiazepines is their positive reinforcement of behaviors previously suppressed by punishment (*16*). The behavioral paradigms used to demonstrate this effect are called "conflict" situations, because the response of the animal is both rewarded (for example, with food) and punished (for example, with electric shock). Under these conditions, untreated animals will normally be influenced by punishment and will suppress responses for the rewarding stimulus. The administration of benzodiazepines characteristically increases the behavioral response for the rewarding stimulus during conditions of punishment. Since the neuronal mechanisms that mediate the suppressing effects of punishment are believed to be inhibitory, the benzodiazepines have been thought of as "disinhibitors" of suppressed behavior. Regardless of the emotional tone one envisions for this type of "conflict" in animals, the major clinical effects of the benzodiazepines also involve a reduction in the behavioral consequences of frustration, fear, and punishment. Thus, the pharmacological activity of the benzodiazepines in the conflict situation may represent a close behavioral corollary to their anxiolytic activity in man.

The benzodiazepines display a variety of other behavioral effects (*17*), but the specificity of these effects for the benzodiazepines and their relation to anxiolytic activity in man (*17*) are not well established. An example is the antiaggressive or "taming" effects observed when the benzodiazepines are administered to aggressive animals (*17*). Although this effect occurs at doses that do not impair motor behavior, similar and more potent antiaggressive activity can be demonstrated for many other drugs, including the phenothiazines, stimulants, opiates, and anticholinergic agents. In addition, like all psychotropic drugs, the benzodiazepines produce undesirable effects (for example, ataxia) that may (or may not) be related to their therapeutic effects. Therefore, studies designed to elucidate the neurochemical mechanisms mediating the anxiolytic action of these drugs must also consider concomitant neurochemical events responsible for unrelated behavioral effects.

Summary. Investigation of the actions of the benzodiazepines has provided insights into the neurochemical mechanisms underlying anxiety, seizures, muscle relaxation, and sedation. Behavioral, electrophysiological, pharmacological, and biochemical evidence indicates that the benzodiazepines exert their therapeutic effects by interacting with a high-affinity binding site (receptor) in the brain. The benzodiazepine receptor interacts with a receptor for γ-aminobutyric acid, a major inhibitory neurotransmitter, and enhances its inhibitory effects. The benzodiazepine receptor may also interact with endogenous substances and several naturally occurring compounds, including the purines and nicotinamide, are candidates for this role. Both the purines and nicotinamide possess some benzodiazepine-like properties in vivo, although further work will be required to confirm their possible roles as endogenous benzodiazepines.

Electrophysiological Studies

One approach to understanding the cellular mechanism of action of benzodiazepines is to examine the effects of these compounds on known transmitter systems within the CNS. The systemic administration of benzodiazepines has been found to affect the concentrations of most putative neurotransmitters [including norepinephrine, dopamine, 5-hydroxytryptamine, acetylcholine, glycine, and γ-aminobutyric acid (GABA)] (*18–20*). While these changes may be related to the behavioral effects of the benzodiazepines (*19–21*), the primary neural pathway responsible for the clinical effects of the benzodiazepines is not clear.

Investigation of the systemic and direct (by microiontophoretic application) effects of benzodiazepines on neuronal activity of identified cells innervated by known transmitter systems (*22*) is one approach to demonstrating the interactions between the benzodiazepines and putative neurotransmitters. Use of these electrophysiological procedures led several authors to suggest that at least part of the actions of benzodiazepines result from a specific interaction with the inhibitory transmitter GABA (*23, 24*). Early experiments demonstrated that diazepam could potentiate presynaptic inhibition in the cat spinal cord (*25*) where GABA is assumed to be the neurotransmitter (*26*). This study (*25*) showed a selective sensitivity of the benzodiazepines for GABA-mediated events; postsynaptic inhibition in this same region, presumed to be mediated by glycine, was unaffected by benzodiazepines. The enhancement of presynaptic GABA-mediated inhibition by benzodiazepines in the spinal cord was confirmed in other laboratories (*24, 27, 28*) and found to occur in other regions, including the sympathetic ganglia (*29*) and the cuneate nucleus (*30*), that is, a group of cells in the medulla. In the cuneate nucleus, both pre- and postsynaptic inhibition are mediated in part by GABA (*26*) and both of these inhibitory events are enhanced by benzodiazepines (*30*). Facilitation of GABA-mediated postsynaptic inhibition has also been observed in the cerebral cortex, substantia nigra, hippocampus, and cerebellar Purkinje cells (*24, 31, 32*). Synergistic actions of benzodiazepines with iontophoretically applied GABA have been demonstrated in many different neuronal systems: cerebral cortex (*33*), cerebellar Purkinje cells (*34*), cerebellar slice preparations (*35*), dorsal raphe cells (*36*), tuberal hypothalamic cell culture (*35*), chick spinal cell cultures (*37*), and fetal rat spinal cord cultures (*38*).

Benzodiazepines have also been reported to antagonize GABA-mediated inhibition in Deiters nucleus in the medulla, in the cerebellum (*39*), and in cultured cerebellar Purkinje cells (*40*). These seemingly opposite effects of ben-

zodiazepines on GABA function may be explained by the recent observation that benzodiazepine effects are dose-dependent (*38*). Low iontophoretic doses of benzodiazepines enhance GABA-mediated inhibition in spinal cord cultures, whereas higher doses of benzodiazepines antagonize the same GABA responses. Benzodiazepine effects on GABA-mediated events have been specifically blocked by antagonists of GABA, such as picrotoxin (*23, 27, 30, 34-36*) and bicuculline (*24, 28, 32, 41, 42*), and have been enhanced by agents such as aminooxoacetic acid that increase GABA concentrations in the brain (*30, 36*). In addition, inhibition of GABA synthesis abolishes the facilitating effects of diazepam on GABA, at least at presynaptic sites (*23, 30*). These studies confirm pharmacologically that GABA and the benzodiazepines interact.

The specificity of the benzodiazepines in enhancing GABA-mediated effects on neurons is indicated by the lack of effects on synaptic events known to be mediated by other transmitters. No effects on glycine-mediated transmission (*25, 43*), on iontophoretically applied glycine (*35-38, 42*), or on strychnine sensitivity (*36, 42, 43*) have been observed. In addition, responses to microiontophoretically applied 5-hydroxytryptamine in dorsal raphe cells (*36*) or to iontophoretically applied norepinephrine in cerebellar Purkinje cells (*34*) were not affected by benzodiazepines. Direct iontophoretic interactions of benzodiazepines with other putative transmitters have not been reported.

Thus the electrophysiological data support the hypothesis that benzodiazepines modulate GABA-mediated synaptic events and that, at pharmacologically relevant doses, these drugs potentiate GABA-mediated inhibition in the CNS. Since this potentiation has been observed in many areas of the brain and spinal cord, it seems to be a general effect of the benzodiazepines in nervous tissue.

The molecular mechanisms responsible for potentiation of GABA-mediated inhibition by benzodiazepines are not yet clear. Potentiation might occur by: (i) a direct activation of GABA receptors, (ii) an increase in presynaptic GABA release, (iii) an inhibition of GABA removal from its receptor site, or (iv) an alteration in the postsynaptic response to GABA. That the benzodiazepines do not exactly mimic the effects of GABA (in spite of facilitated GABA-mediated events) suggests that the first possibility can be excluded (*34, 36-38, 44*). In addition, in studies where GABA has been applied by ionotophoresis, the potentiation of the GABA-mediated response by benzodiazepines does not seem to be due to increased presynaptic release of GABA (*34, 36-38*). Evidence also fails to support the idea that benzodiazepines block the uptake (*35, 36*) or catabolism (*30, 36*) of GABA. Thus, alterations of postsynaptic responses to GABA remain the most likely possibility. Yet even this hypothesis may not be sufficient to explain completely every pharmacological action of the benzodiazepines. Many behavioral studies have failed to demonstrate a synergistic relation between GABA and benzodiazepines. GABA mimetics, although potent as anticonvulsants, fail to act behaviorally in the conflict test (*16*). Furthermore, it seems unlikely that changes in the response to GABA in the spinal cord could be relevant to the antianxiety properties of the benzodiazepines, although such changes might be related to the ataxia produced by benzodiazepines.

The electrophysiological effects of benzodiazepines have been compared with representative compounds from other classes of drugs that exhibit one or more of the pharmacological actions displayed by benzodiazepines. Such comparisons have been made with the barbiturates (*44, 45*), the anticonvulsant diphenylhydantoin (*46*), and the major tranquilizer chlorpromazine (*28*). However, these studies have not yielded definitive information about unique electrophysiological characteristics of the benzodiazepines. Since it has been estimated that GABA probably mediates transmission in at least 30 percent of brain synapses (*47*), it is likely that any modification of this transmitter could result in extensive repercussions throughout the CNS (*44*).

Table 1. Inhibition (K_i) by various benzodiazepines of [^{3}H]diazepam binding to rat brain membranes and the therapeutic doses of these drugs in man. These data show that there is a correlation between the ability to inhibit binding and therapeutic effects in man. However, the data do not take into account drug metabolism or penetration into the brain. With a more extensive series of drugs a correlation of 0.83 ($P < .005$) was observed (*50*). It should be noted that the values for the dose causing drug effect in the majority of subjects are higher than the average clinical dose.

Compound	K_i (n*M*)*	Dose causing drug effect in majority of subjects (mg/day)†
Clonazepam	2	7.5
Flunitrazepam	3	6
Diazepam (Valium)	9	75
Flurazepam (Dalmane)	16	100
Nitrazepam	19	30
Bromazepam	30	60
Chlordiazepoxide (Librium)	570	100
RO5-5807	2,600	160
RO5-4864	> 100,000	Not active

*Data from (*49*). †Data from (*6*).

Benzodiazepine Receptors in the Brain

The use of radioactively labeled compounds of high specific activity in studies of brain receptor binding has rapidly advanced our knowledge of the biochemical mechanisms of action of the benzodiazepines (*48*). By using ^{3}H-labeled diazepam and ^{3}H-labeled flunitrazepam, it has been possible to demonstrate that there are high-affinity binding sites in mammalian brain that fulfill many of the criteria of pharmacological receptors for these compounds; binding to these sites in vitro is rapid, reversible, stereospecific, and saturable (*49*). The finding of significant correlations between the behavioral and clinical potency of a series of benzodiazepines and their ability to displace [^{3}H]diazepam binding from mammalian brain membrane preparations in vitro (*49, 50*) also suggests that the benzodiazepine binding site may mediate the pharmacological actions of these drugs (Table 1). Significant correlations have also been reported between the ability of benzodiazepines to displace [^{3}H]diazepam from its binding site and their ability to inhibit pentylenetetrazole-induced convulsions and increase response rates after punishment in a conflict test. These last two characteristics are indicative of anxiolytic properties (*51*).

Benzodiazepine receptors occur in the brain (*49*), and a high density of specific binding sites has been associated with synaptosomal membranes (*49*). A high-affinity binding site for some of the benzodiazepines has also been found in the kidney, liver, lung, and several tissue-culture cell lines; however, the pharmacological specificity of this site is different from the analogous brain sites. Furthermore, the affinity of various benzodiazepines for the peripheral binding site does not appear to be correlated with the clinical or behavioral effects of these drugs (*49, 52*). Although initial studies on the distribution of brain benzodiazepine receptors indicated that they occurred predominantly in glial cells (*53*), subsequent lesion studies (*54*), studies of mutant mice with neurological defects (*55*), and autoradiographic studies (*56*)

now support the view that they occur on neurons. In addition, autoradiographic studies (*56*) have demonstrated significant differences in the regional distribution of these receptors in rat, mouse, and human brain. In both rat and human brain, for example, there is a high density of receptors in the molecular layer of the cerebellum; but compared with that of the human, the granule cell layer of the rat cerebellum has a relatively reduced concentration of receptors. The lack of receptors in white matter was a consistent finding in all species. Species differences in receptor distribution point out the difficulties in ascribing a particular anatomical location to drug action and in extrapolating the results of behavioral and pharmacological studies from animals to humans. The widespread distribution of benzodiazepine receptors in the nervous system contrasts with the more discrete localization of other receptors, such as the opiate receptor (*57*), and support the evidence from behavioral and electrophysiological studies that the benzodiazepines interact at many levels of the brain to produce their effects.

Consistent with the widespread distribution of these receptors, biochemical evidence also indicates that the benzodiazepines affect several neuronal pathways. The neurotransmitter whose function has been most closely associated with the benzodiazepines is GABA (*23, 24*), and the electrophysiological evidence described earlier is consistent with a facilitatory interaction of benzodiazepines with GABA-mediated transmission. Initial binding studies (*49, 50*) did not indicate any interaction between GABA receptors and the benzodiazepine receptor; however, it is now generally believed that such an interaction occurs (*58-60*). Enhanced binding of [^{3}H]diazepam is obtained when either GABA or one of its analogs is included in the binding assay (*58, 59*). The enhanced binding of ^{3}H-labeled benzodiazepines in the presence of GABA is due to an increase in the affinity of the benzodiazepine receptor for its ligand with no alteration in receptor number. The GABA-like antagonist bicuculline stereospecifically antagonizes the increase in [^{3}H]diazepam binding caused by GABA and can decrease control binding (probably because of antagonism of endogenous GABA) (*58*) (Table 2).

This finding has been confirmed (*60*) and extended to demonstrate that the magnitude of GABA potentiation of benzodiazepine binding varies regionally in the brain. This may be because of heterogeneity in either the benzodiazepine receptors (*61*) or the GABA receptors (*62*), or to altered interactions between the two receptors. Recently, heat inactivation has been used to define two benzodiazepine receptors with different thermostability properties (*61*). These two receptors also interact in different ways with several anxiolytics that are not benzodiazepines (*61, 63*). Actual differences in the primary structure of either GABA or benzodiazepine receptor proteins have not yet been shown and unequivocal demonstration of multiple receptors has not been accomplished. Such a demonstration may require purification of these receptors.

Table 2. Effect of GABA and related drugs on [^{3}H]diazepam binding.

Drug addition	Specific [^{3}H]diazepam binding as a percentage of control
None	100 ± 2
GABA (5 μM)	123 ± 2*
(+)-Bicuculline methiodide (100 μM)	63 ± 1†
GABA plus (+)-bicuculline methiodide	64 ± 1‡
Muscimol (10 μM)	127 ± 2§
GABA plus muscimol	126 ± 2¶

*$P < .01$ ($N = 4$) compared to control. †$P < .001$ ($N = 4$) compared to control. ‡$P < .001$ ($N = 4$) compared to GABA alone. §$P < .001$ ($N = 4$) compared to control. ¶Not different from GABA alone.

Another approach to studying interactions between GABA receptors and benzodiazepines has focused on alterations in GABA binding induced by benzodiazepines (*64*). Initial studies of GABA binding indicated that treatment of brain membranes with the detergent Triton X-100 unmasked a high-affinity GABA receptor by removal of GABA-modulin, that is, an endogenous modulator of GABA binding (*65*). In addition to inhibiting GABA binding, GABA-modulin appears to inhibit competitively benzodiazepine binding (*64*). When GABA-modulin is added to Triton-treated membranes it inhibits the high-affinity GABA binding, and this effect is stereospecifically antagonized by benzodiazepines. Thus, a model may be constructed wherein GABA-modulin interacts with both GABA and benzodiazepine receptors as a part of a postsynaptic GABA-benzodiazepine-ionophore complex to keep the GABA receptor in a low-affinity state.

Interactions between the benzodiazepine receptor and anions have also been demonstrated. Chloride, bromide, iodide, nitrite, and thiocyanate, but not fluoride, enhance the binding of [^{3}H]-diazepam, and such data suggest a role for anions in regulating the affinity of the benzodiazepine binding site (*66*). It is interesting that the specificity of this effect has been correlated with the ability of anions to penetrate activated postsynaptic motoneurons of the cat. This correlation has lent additional support to the speculation that the benzodiazepine receptor occurs in close proximity to a chloride ionophore.

The benzodiazepine receptors are associated with GABA receptors and a chloride ionophore in many lower invertebrates might indicate a supramolecular complex that is phylogenetically old. However, studies of the binding of [^{3}H]diazepam to invertebrate and hemichordate brain have failed to reveal binding sites with pharmacological properties similar to those in mammalian brain (*50*). Although binding sites for diazepam have been demonstrated on intact schistosomes (blood flukes), these sites have a lower affinity and a different pharmacological profile from those in mammalian brain (*67*). The occurrence of such sites in schistosomes may indicate that the mammalian brain site has undergone significant phylogenetic modification. Ontogenetically, the benzodiazepine receptor can be found at 14 days of gestation in the rat (*68, 69*), and the number of sites increases in parallel to the GABA receptor; some investigators have found that the activation of [^{3}H]diazepam binding by GABA is greater in fetal and newborn brain than in the adult (*69*).

In an attempt to understand the interactions between the various proteins in the GABA-benzodiazepine supramolecular complex, investigators in several laboratories have begun to purify GABA receptors (*70*), GABA-modulin (*64*), and the benzodiazepine receptor (*71*). The benzodiazepine receptor is a 200,000-dalton protein (*71*), and electrophoretic studies suggest subunits of 50,000 daltons (*72*). GABA-modulin is a thermostable, acidic protein of 15,000 daltons (*64*), whereas the GABA receptor itself has been reported to have a molecular weight of 900,000 (*70*). Benzodiazepine binding to a detergent solubilized receptor is highly temperature-dependent (highest affinity at 4°C); therefore, binding appears to be, in part, entropy driven (*71*). The availability of several irreversible ligands (compounds which form covalent bonds with the receptor), including irazepine (*73*), and the recently discovered photoaffinity (light-activated cross-linking) property of flunitrazepam (*72*) should facilitate purification of the benzodiazepine receptor. The irrevers-

ible ligands may also distinguish between the possible subtypes of benzodiazepine receptors, because the reactivity of the receptor's active site would be anticipated to differ for genetically distinct receptor molecules or for receptors in different membrane environments.

Intracellular Events and Model Systems

Binding to a receptor is only the first step in a sequence of events that is initiated by a drug or transmitter. In the case of the benzodiazepines, either activation or priming of an anion ionophore is a consequence of binding to the receptor. In the cerebellum, the activity of Purkinje cells is related to their content of guanosine 3′,5′-monophosphate (cyclic GMP) (*74*); activation of GABA receptors results in a net decrease in the firing of the cells and a decrease in cyclic GMP formation. Diazepam blocks the increase in the cyclic GMP content of the cerebellum after GABA synthesis is inhibited, and the effects of diazepam seem to be mediated through interactions with the GABA-benzodiazepine supramolecular complex (*64, 65, 74*).

High-affinity benzodiazepine binding sites have been identified on cultured cells of neural origin (*52, 75*). The relation between the receptors found on these lines and the brain receptors is unclear, since there are substantial pharmacological differences between the two types of receptor (*52, 76, 77*). The lipid environment of the receptor may play a role in determining the receptor's specificity (*75*); thus, the recent demonstration that occupation of benzodiazepine receptors activates phosphatidyl choline synthesis may have important implications for the functioning of the benzodiazepine and GABA receptors in the membrane (*77*). An interaction of benzodiazepine receptors with phospholipids may also be relevant to GABA activation of benzodiazepine binding and enhancement of chloride transport (*75*). A promising model for the study of the brain-specific benzodiazepine receptors may be a primary cell culture derived from spinal cord where both electrophysiology (*38*) and biochemistry (*78*) can be studied in the same preparation.

Receptor Studies in vivo

Investigators have examined the effects of several nonphysiological conditions on benzodiazepine binding in vitro and they have found, for example, that binding is highly temperature-dependent in vitro and that the amount bound at 37°C is much less than binding obtained at 0° to 4°C, the typical incubation temperature (*49*). Thus, serious questions can be raised concerning the relevance of the studies of binding in vitro to the mechanisms operating in vivo. To demonstrate benzodiazepine receptors under physiological conditions, several workers have administered radioactive benzodiazepines in vivo and examined the binding to neuronal and various peripheral tissues (*79–82*). Such procedures have been used previously in studies of opiate and β-adrenergic receptor binding (*83*).

In studies with rats, maximum binding of [^{3}H]diazepam occurred extremely rapidly after the drug was administered intravenously. The rank order of the amounts of displaceable binding between brain regions as determined by binding assays in vitro correlated closely with that observed in vivo (*79–82*). After intravenous administration, [^{3}H]diazepam also accumulated in peripheral organs that included the kidney, liver, and skeletal muscle (*81*). However, the amount of binding in these tissues was less than that observed in brain tissue. In addition, binding of [^{3}H]diazepam in vivo to peripheral tissues lacked the pharmacological specificity and stereospecificity observed in the brain and spinal cord (*81*). Treatment of animals with biologically active benzodiazepines prevented the binding of intravenously injected [^{3}H]-diazepam. In contrast, when animals were first treated with pharmacologically inactive benzodiazepines (*79–82*), including inactive stereoisomers (*81*), no significant alterations occurred in the binding of [^{3}H]diazepam in vivo. These studies in vivo support the view that pharmacologically relevant benzodiazepine binding is restricted to the CNS (*49*).

Treatment of animals with the GABA agonist muscimol and the GABA catabolic inhibitor aminooxoacetic acid also enhances [^{3}H]diazepam binding in vivo (*82*). The magnitude of the GABA-mediated enhancement of [^{3}H]diazepam binding in vivo was similar to the increase observed with an assay in vitro (*58*). These studies suggest a strong correlation between data obtained by direct labeling of benzodiazepine receptors in vivo and by assay procedures in vitro, and thus support the validity of the technique in vitro for the study of the benzodiazepine receptor.

If these binding sites for benzodiazepines can modulate the pharmacological effects of benzodiazepines, altered input to these receptors may affect either the number or character of these binding sites, analogous to changes observed in other receptor systems (*84*). For example, although the effects of long-term benzodiazepine treatment on binding remains controversial (*85*), two laboratories (*86*) have reported significant decreases in benzodiazepine binding after different regimens of daily drug administration. These changes were reported as decreases in the total number of binding sites without alterations in affinity for benzodiazepines. Since comparable treatment of rats with barbital did not affect benzodiazepine binding (*86*), these data may suggest a specific response to long-term receptor occupation, rather than a generalized adaptation to chronic CNS depression.

The use of animal models has also provided information about the cellular localization and physiological function of the benzodiazepine receptor. In an attempt to determine the cellular distribution of the benzodiazepine receptor, [^{3}H]diazepam binding was studied in the cerebella of mutant mice with neurological defects. Several groups (*55*) have demonstrated decreases in the number of benzodiazepine receptors in the cerebellum of the "nervous" mouse. This mutant undergoes a spontaneous degeneration of Purkinje cells that is fully apparent at 60 to 70 days after birth (*87*). Young animals that have not undergone this degeneration have a full complement of benzodiazepine receptors, as do heterozygote adults that possess a nervous (*nr*) gene but not phenotypical expression of the trait (*55*). This loss in benzodiazepine receptors is especially remarkable considering the relative concentration of Purkinje cells compared to the total neuronal population of the cerebellum (<<1 percent of cerebellar neurons). The mutant mouse known as "staggerer," which lacks synaptic spines on Purkinje cell dendrites (where they would normally synapse with granule cell parallel fibers), also has a reduced number of cerebellar binding sites (*88*). In contrast, "weaver" mutants, which undergo granule cell degeneration (*89*), have a relative enrichment in cerebellar benzodiazepine binding sites (*54*). No change in the affinity of the receptor for tritiated benzodiazepines was observed in any of the neurologic mutants. Lesions of the cerebellum brought about by application of kainic acid have also been reported to decrease benzodiazepine binding. In animals with lesions, the reduction in binding is reported to be due to a decrease in the total number of binding sites without a change in affinity (*54*). Concomitant changes in

GABA receptor binding in mutants or in rats with lesions have also been observed (*54*). These data suggest that benzodiazepine receptors in the cerebellum are concentrated at or near Purkinje cells.

The benzodiazepine receptor has been implicated in both the anxiolytic and anticonvulsant actions of the benzodiazepines. Recently it was reported that two strains of rats, bred selectively for high and low fearfulness, have significantly different densities of brain benzodiazepine receptors. Since these two strains differ in their emotionality, differences in benzodiazepine receptor number may be physiologically important in the regulation of anxiety (*90*). Alterations in benzodiazepine binding have also been reported in a spontaneously epileptic baboon (*61*), and an increase in the number of benzodiazepine receptors was observed in rats subjected to either electrically or chemically induced seizures (*91*). The changes in receptor number after seizures are comparable to the changes observed in other CNS receptors after treatments designed to alter neuronal input (*92*). However, the rapid onset (within minutes) of the changes in benzodiazepine receptors and their return to control levels (within 1 hour) is remarkable; such changes in other central receptors usually occur over a period of days. Experimental anxiety in rats—produced by a conflict situation or foot shock—decreased [^{3}H]diazepam binding in frontal cortex (*93*). It is not known if these changes result from a decrease in the affinity or the number of receptors. These anxiety-induced changes were also rapid, occurring after a standard 5-minute conflict situation or a brief exposure to an electrified floor grid. Significant increases in benzodiazepine binding were recently observed to occur within 1 hour after the administration of the anticonvulsant agent diphenylhydantoin (*94*).

The relation between benzodiazepine receptors and the anticonvulsant effects of the benzodiazepines has been examined in vivo (*95*). A high degree of correlation was observed between the number of drug-occupied benzodiazepine receptors and the effects of diazepam in preventing convulsions after pentylenetetrazole administration. Furthermore, only 20 to 30 percent of the receptors measured in vitro appear to be occupied when animals are fully protected against seizures. It was also reported that 10 to 20 percent of benzodiazepine receptors must be occupied in order to elicit the maximum anxiolytic effects of diazepam in the conflict test (*51*). These findings directly implicate the benzodiazepine receptor in at least two pharmacological actions of the benzodiazepines and suggest that small changes in receptor number or affinity may have profound behavioral consequences. Despite evidence that changes in benzodiazepine binding occur in vivo and are associated with distinct behavioral and pharmacological consequences, the functional significance of these changes are unknown.

Endogenous Ligands

The existence of benzodiazepine receptors and the inability of a wide variety of known transmitters to inhibit [^{3}H]diazepam binding in vitro suggest that the brain may contain an unidentified endogenous ligand. Since there is no suitable bioassay for testing many samples in vitro, the ability of brain fractions to inhibit [^{3}H]diazepam binding was used to search for endogenous benzodiazepine ligands. Isolation of endogenous ligands was initially accomplished by using acidified acetone or methanol extracts of bovine (*96*) or rat brain (*97*). After further purification (*96*) several discrete inhibitory substances were obtained. The inhibitory activity of these active fractions was dialyzable, heat stable, and not degraded by proteolytic enzymes. These inhibitors were identified as the purines inosine and hypoxanthine (*98*), and this identification was confirmed by a radioimmunoassay with antibodies specific for diazepam as well as by mass spectroscopy (*99*). However, the affinity of these purines for the benzodiazepine receptor is many orders of magnitude lower than that of the benzodiazepines (the inhibition constants of inosine and hypoxanthine approach 1 m*M* compared to 5 n*M* for diazepam). Despite this relatively low affinity, some evidence suggests that these or related purines may be endogenous modulators of the benzodiazepine receptor. Intraventricular administration of inosine and 2′-deoxyinosine increases the seizure latency induced by pentylenetetrazole, whereas closely related purines that do not compete with [^{3}H]diazepam for receptor sites do not affect seizure latency (*100*). Prolongation of seizure latency appears to be both dose- and time-dependent. The effects of inosine are transitory, and this may be partially explained by either the rapid loss of intraventricularly injected inosine from the CNS or its rapid metabolism (*101*). The electrophysiological effects of the flurazepam have been compared to those of inosine in primary cultures of mouse spinal cord neurons. Iontophoretic application of inosine elicits two types of transmitter-like effects: a rapidly desensitizing excitatory response and a nondesensitizing inhibitory response (*102*). Flurazepam produces a similar excitatory response that shows cross-desensitization with the excitation produced by inosine. The slow inhibitory response observed with inosine was blocked by flurazepam. These results provide electrophysiological evidence that inosine can activate two different conductances on spinal neurons and that flurazepam activates one of these conductances and antagonizes the other. Recently it has been shown that modification of the imidazolopyrimidine (purine) nucleus results in compounds with a high affinity for the benzodiazepine receptor and potent benzodiazepine-like effects in animal screening tests. Increasing the lipid-like character of the purine molecules also results in a marked increase in their affinity (more than 100-fold) in vitro for the benzodiazepine receptor (*103*).

Thromboxane A_2, a short-lived intermediate derived from prostaglandin endoperoxides, has been suggested as a candidate for an endogenous ligand of the benzodiazepine receptor (*104*). Evidence from rat vascular preparations sensitive to benzodiazepines indicates that the vasopressor effects of thromboxane A_2 are antagonized by both diazepam and chlordiazepoxide. The examination of this compound as a possible endogenous ligand must be further evaluated both in a test system containing brain-specific receptors and with a larger series of benzodiazepines.

Competitive inhibition of [^{3}H]diazepam binding by a factor (40,000 to 70,000 daltons) has also been reported (*105*). This factor is heat stable but susceptible to proteolytic degradation. An endogenous ligand of this molecular weight would be a neurohormone of extraordinary size, and it has been suggested that the factor may represent a precursor of a smaller physiologically active ligand.

Nicotinamide has also been isolated from acetone or perchloric acid extracts of bovine and rat brain (*101*). The affinity of nicotinamide for the benzodiazepine receptor appears to be four to five times lower than the affinities reported for the purines. However, it has also been reported that nicotinamide enhances presynaptic inhibition in dorsal root preparations of cat cord, an effect that is reversed by bicuculline. These effects were mimicked by intravenous injection of diazepam. Nicotinamide partially restored punishment-suppressed behavior

in rats, and in large doses blocked 3-mercaptoproprionic acid-induced seizures. These observations suggest that despite its low affinity in binding assays, nicotinamide must also be considered as an endogenous benzodiazepine-like substance.

Most recently, a peptide isolated from fractions of small intestine and bile duct of the rat has been reported to inhibit competitively [^{3}H]diazepam binding (*106*). This peptide is resistant to proteolysis and has a molecular weight of 16,000. Its apparent affinity for the receptor is quite high and, although it has not yet been isolated from brain, immunohistochemical studies suggest that it is present in the deep layers of the cerebral cortex.

While all of the compounds discussed in this section are candidates for the role of endogenous ligand, there is no compelling evidence for the exclusive role of any one of them. An additional possibility is that receptor occupation by GABA or benzodiazepine, or both, results directly in conformational changes of an adjacent ionophore. Such alterations could result in permeability changes to ions regulating neuronal activity (*107*). A protein modulator that interacts at the high-affinity benzodiazepine binding site in the GABA-benzodiazepine-ionophore complex (*64*) could also interact during the conformational change. Thus, the existence of an additional transmitter substance or endogenous ligand may not be required to explain the pharmacological properties of the benzodiazepines.

Clinical Implications, Applications, and Conclusions

Over the past few years it has become apparent that many psychotropic drugs function by mimicking, antagonizing, or enhancing the central effects of endogenous neurohumoral substances (*48*). It is not surprising, therefore, that a similar mechanism would be sought for the benzodiazepines. Demonstration of high-affinity binding sites for the benzodiazepines in brain, coupled with compelling pharmacological data linking occupation of these sites to the drugs' behavioral effects, indicate that these specific discriminatory sites represent the places where the benzodiazepines exert their effects.

These findings have had almost immediate practical application. For example, it is feasible and less expensive to screen large numbers of potential antianxiety and anticonvulsant drugs by using their ability to inhibit [^{3}H]benzodiazepine binding in vitro; active compounds can then be tested behaviorally. Two new nonbenzodiazepine compounds (*63, 108*), both with a relatively high affinity for the receptor and with potent benzodiazepine-like behavioral activity in animal screening tests, have already been developed in this way. Furthermore, one of these compounds (*63*), a triazolopyridazine, apparently lacks many of the undesirable side effects (for example, potentiation of alcohol-induced narcosis) observed with the benzodiazepines themselves. Another clinical application of benzodiazepine binding involves the development of a sensitive, rapid, and inexpensive "radioreceptor assay" to measure benzodiazepines in biological samples, including blood (*109*). This method, in contrast to more complicated analytical procedures, can be carried out in most clinical settings and should prove useful in monitoring the concentrations of benzodiazepines in the blood for therapeutic or toxicological procedures.

The findings that both benzodiazepine binding and the electrophysiological actions of benzodiazepines are enhanced by GABA may also have important applications, such as in the clinical management of seizure disorders. It is possible that the efficacy of benzodiazepines in long-term treatment of human epilepsy may be improved by concomitant administration of centrally active compounds that mimic the effect of GABA. The development of such compounds could also be facilitated by studies in vitro of their potency in enhancing [^{3}H]benzodiazepine binding. One such compound has already been reported to enhance benzodiazepine binding both in vitro and in vivo, and seems to affect behavior in animals (*63*).

Identification of an endogenous substance that interacts with the benzodiazepine receptor would undoubtedly have broad and important implications in neurobiology. Although none of the candidates to date satisfy all criteria, the purines and nicotinamide do have benzodiazepine-like properties in vivo. It is possible, therefore, that these or similar substances may be physiological modulators of the benzodiazepine receptor.

Thus, this multidisciplinary approach to the molecular pharmacology of the benzodiazepines can provide new insights into the basis of anxiety and promote the development of better drugs for treating one of man's most common psychiatric disorders.

References and Notes

1. C. D. Spielberger, *Anxiety and Behavior* (Academic Press, New York, 1976); E. E. Levitt, *The Psychology of Anxiety* (Bobbs-Merrill, Indianapolis, 1967); M. Lader and I. Marks, *Clinical Anxiety* (Heinemann Medical Books, London, 1971).
2. L. H. Sternbach, L. O. Randall, S. R. Gustafson, in *Psychopharmacological Agents*, M. Gordon, Ed. (Academic Press, New York, 1964), vol. 1, p. 137; L. O. Randall, W. Shallek, L. H. Sternbach, R. Y. Ning, *ibid.* (1974), vol. 3, p. 175.
3. *Sleeping Pills, Insomnia and Medical Practice, Report of a Study of the Institute of Medicine* (National Academy of Sciences, Washington, D.C., 1979).
4. This estimate is based on an average prescription for 10 units of 10 milligrams each. (This is a conservative estimate, since the unit sizes of flurazepam are 15- and 30-milligram tablets.) It also does not take into consideration other benzodiazepines marketed, including lorazepam and combination tablets of chlordiazepoxide with other agents, such as amitriptyline.
5. D. J. Greenblatt and R. I. Shader, *Benzodiazepines in Clinical Practice* (Raven, New York, 1974); D. J. Greenblatt, *Clin, Pharmacol. Ther.* **21**, 497 (1977); E. M. Sellers, *Can. Med. Assoc. J.* **118**, 1533 (1978); K. Frennsgaard, *Acta Psychiatr. Scand.* **53**, 105 (1976); see also (*11*).
6. G. Zbinden and L. O. Randall, *Adv. Pharmacol.* **5**, 213 (1967).
7. G. B. Fink and E. A. Swinyard, *J. Pharm. Sci.* **51**, 548 (1962).
8. S. C. Kaim and I. N. Rosenstein, *Dis. Nerv. Syst.* **21** (Suppl.), 46 (1960); G. F. Rossi, G. DiRocco, G. Maira, M. Meglio, in *The Benzodiazepines*, S. Garattini, E. Mussin, L. O. Randall, Eds. (Raven, New York, 1973), p. 461.
9. A. S. Lippa, D. Nash, E. Greenblatt, in *Anxiolytics*, S. Fielding and H. Lal, Eds. (Futura, Mount Kisco, N.Y., 1979), p. 41.
10. M. E. Goldberg, A. A. Manian, D. H. Efron, *Life Sci.* **6**, 481 (1967).
11. The question of whether tolerance develops to the therapeutic effects of the benzodiazepines is somewhat controversial. Although the long-term efficacy of the benzodiazepines as anticonvulsants in certain seizure disorders decreases over time (*12*), there is little evidence that tolerance develops to their anxiolytic effects. Nevertheless, clinical studies of tolerance are difficult to carry out because anxiety is episodic. Similarly, it is also unclear as to whether patients who abuse benzodiazepines by increasing their dose are doing so because of the development of tolerance. The use and abuse of benzodiazepines was recently the subject of a congressional hearing ["Use and Misuse of Valium," transcript of the congressional hearing (Government Printing Office, Washington, D.C., in press)].
12. A. S. Lippa, E. N. Greenblatt, R. W. Pelham, in *Animal Models in Psychiatry and Neurology*, I. Hanin and E. Usdin, Eds. (Pergamon, New York, 1978), p. 279; L. Juhasz and W. Dairman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 377 (1977).
13. L. O. Randall, W. Schallek, G. A. Heise, E. F. Keith, R. E. Bagdon, *J. Pharmacol. Exp. Ther.* **129**, 163 (1960).
14. W. Schlosser, *Arch. Int. Pharmacodyn. Ther.* **104**, 93 (1971); S. H. Nagai, D. T. C. Tseng, S. C. Wang, *J. Pharmacol. Exp. Ther.* **153**, 344 (1966); T. C. Tseng and S. C. Wang, *ibid.* **178**, 350 (1971).
15. J. T. Hamilton, *Can. J. Physiol. Pharmacol.* **45**, 191 (1971).
16. I. Geller, J. T. Kulak, J. Seifter, *Psychopharmacologia* **3**, 374 (1962); L. Cook and A. C. Catania, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 818 (1964); L. Cook and A. Davidson, in *The Benzodiazepines*, S. Garattini, E. Mussini, L. O. Randall, Eds. (Raven, New York, 1973), p. 327.
17. S. O. Glick, in *Behavioral Pharmacology*, S. O. Glick and J. Goldfarb, Eds. (Mosby, St. Louis, 1976), p. 344.
18. K. Taylor and R. Laverty, *Eur. J. Pharmacol.* **8**, 296 (1969).
19. H. Corrodi, K. Fuxe, P. Lidbrink, L. Olson, *Brain Res.* **29**, 1 (1971); B. Biswis and A. Carlsson, *Naunyn Schmiedeberg's Arch. Pharmacol.* **303**, 73 (1978); T. Chase, R. Katz, I. Kopin, *Neuropharmacology* **9**, 103 (1970); S. Consolo, S. Garattini, H. Ladinsky, in *Mechanism of Action of Benzodiazepines*, E. Costa

and P. Greengard, Eds. (Raven, New York, 1975), p. 28; S. F. Saad, *J. Pharm. Pharmacol.* **24**, 839 (1972); N. Lippmann and T. Pugsley, *Br. J. Pharmacol.* **51**, 571 (1974); S. Snyder and S. Enna, *Adv. Biochem. Psychopharmacol.* **14**, 81 (1975).
20. K. Fuxe, L. Agnati, P. Bolme, T. Hokfelt, P. Lidbrink, A. Ljungdahl, M. P. la Mora, S. Ogren, *Adv. Biochem. Psychopharmacol.* **14**, 45 (1975).
21. L. Stein, C. Wise, B. Berger, in *The Benzodiazepines*, S. Garattini, E. Mussini, L. Randall, Eds. (Raven, New York, 1973), p. 299; P. Lidbrink, H. Corrodi, K. Fuxe, *Eur. J. Pharmacol.* **26**, 35 (1974); M. Nakamura and H. Fukushima, *Psychopharmacologia* **53**, 121 (1977); P. Jenner, D. Chadwick, E. Reynolds, C. Marsden, *J. Pharm. Pharmacol.* **27**, 707 (1975).
22. Typically, in these techniques an extracellular electrode is used that monitors electrical potential changes of an adjacent neuron. Action potentials are displayed to be resolved into various components and/or counted as unit events (spikes) per specified time interval. Drugs are injected systemically (usually intravenously) or applied in the immediate vicinity of the recorded neuron by means of iontophoretic or pressure ejection techniques. The relevant afferent pathway is then stimulated or the suspected transmitter substance is applied iontophoretically (with a multibarrel electrode positioned near the recorded cell), with the responses being tested both independently and in the presence of the drug under investigation. Drug effects are indicated by changes in firing rates or patterns after drug administration or by changes in responses to afferent stimulation or to sequential applications of putative neurotransmitters (potentiation or inhibition of responses). For technical details, see D. Curtis, in *Physical Techniques in Biological Research*, W. Nastak, Ed. (Academic Press, New York, 1964), vol. 5, pt. A, p. 144; G. Salmoiraghi and C. Stefanis, *Int. Rev. Neurobiol.* **10**, 1 (1967); R. Thompson and M. Pattersen, Eds., *Bioelectric Recording Techniques* (Academic Press, New York, 1973), pt. A, p. 412; F. Bloom, *Life Sci.* **14**, 1819 (1974); A. Podjen and F. Bloom, in *Mechanism of Action of Benzodiazepines*, E. Costa and P. Greengard, Eds. (Raven, New York, 1975), p. 93.
23. E. Costa, A. Guidotti, C. Mao, A. Suria, *Life Sci.* **17**, 167 (1975).
24. W. Haefely, A. Kulesan, H. Mohler, L. Piere, P. Polc, R. Schaffner, in *Mechanism of Action of Benzodiazepines*, E. Costa and P. Greengard, Eds. (Raven, New York, 1975), p. 131.
25. R. Schmidt, E. Vogel, M. Zimmermann, *Naunyn Schmiedeberg's Arch. Pharmacol.* **258**, 69 (1967).
26. D. Curtis and G. Johnston, *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **69**, 97 (1974).
27. W. Stratten and C. Barnes, *Neuropharmacology* **10**, 685 (1971).
28. P. Polc, H. Mohler, W. Haefely, *Naunyn Schmiedeberg's Arch. Pharmacol.* **284**, 319 (1974).
29. A. Suria and E. Costa, *Brain Res.* **50**, 235 (1973).
30. P. Polc and W. Haefely, *Naunyn Schmiedeberg's Arch. Pharmacol.* **294**, 121 (1976).
31. W. Raabe and R. Gumwit, *Epilepsia* **18**, 117 (1977); V. Zakusov, R. Ostrovskaya, V. Markovitch, G. Molodavkin, V. Bylayev, *Arch. Int. Pharmacodyn. Ther.* **214**, 188 (1975); P. Wolf and H. Haas, *Naunyn Schmiedeberg's Arch. Pharmacol.* **299**, 211 (1977); D. Curtis, D. Lodge, G. Johnston, S. Brand, *Brain Res.* **118**, 344 (1976); P. Montarolo, F. Raschi, P. Strata, *ibid.* **162**, 358 (1979).
32. T. Tsuchiya and H. Fukushima, *Eur. J. Pharmacol.* **48**, 421 (1978).
33. S. Kozhechkin and R. Ostrovskaya, *Nature (London)* **269**, 72 (1977).
34. H. Geller, D. Taylor, B. Hoffer, *Naunyn Schmiedeberg's Arch. Pharmacol.* **304**, 81 (1978).
35. K. Okamoto and Y. Sakai, *Br. J. Pharmacol.* **65**, 277 (1979).
36. D. Gallager, *Eur. J. Pharmacol.* **49**, 133 (1978).
37. D. Choi, D. Faub, G. Fischbach, *Nature (London)* **269**, 342 (1977).
38. R. Macdonald and J. Barker, *ibid.* **271**, 563 (1978).
39. F. Steiner and D. Felix, *ibid.* **260**, 346 (1976).
40. B. Gahwiler, *Brain Res.* **107**, 176 (1976).
41. R. Polzin and C. Barnes, *Neuropharmacology* **18**, 431 (1979).
42. A. Dray and D. Straughan, *J. Pharm. Pharmacol.* **28**, 314 (1976).
43. D. Curtis, C. Game, D. Lodge, *Br. J. Pharmacol.* **56**, 307 (1976).
44. W. Haefely, *Agents Actions* **7**, 353 (1977).
45. F. Steiner and P. Hummel, *Int. J. Neuropharmacol.* **7**, 61 (1968).
46. W. Matthews and J. Connor, *Neuropharmacology* **15**, 181 (1976).
47. F. Bloom and L. Iversen, *Nature (London)* **229**, 628 (1971).
48. M. Blecher, *Methods in Receptor Research* (Dekker, New York, 1976), vols. 1 and 2; H. I. Yamamura, S. J. Enna, M. J. Kuhar, *Neurotransmitter Receptor Binding* (Raven, New York, 1978).
49. C. Braestrup, R. Albrechtsen, R. F. Squires, *Nature (London)* **269**, 702 (1977); H. Möhler and T. Okada, *Science* **198**, 849 (1977); C. Braestrup and R. F. Squires, *Proc. Natl. Acad. Sci U.S.A.* **74**, 3805 (1977); H. Möhler and T. Okada, *Life Sci.* **20**, 2101 (1977); H. B. Bosmann, D. P. Penney, K. R. Case, P. Di Stefano, K. Averill, *FEBS Lett.* **82**, 368 (1977); *ibid.* **87**, 199 (1978); R. C. Speth, G. J. Wastek, P. C. Johnson, H. Yamamura, *Life Sci.* **22**, 859 (1978).
50. C. Braestrup and R. Squires, *Br. J. Psychiatry* **133**, 249 (1978); H. Mohler and T. Okada, *ibid.*, p. 261.
51. H. Mohler, T. Okada, J. Ulrich, P. H. Heitz, *Life Sci.* **22**, 985 (1978); A. S. Lippa, C. A. Klepner, L. Younger, M. C. Sano, W. V. Smith, B. Beer, *Pharmacol. Biochem. Behav.* **9**, 853 (1978).
52. P. J. Syapin and P. J. Skolnick, *J. Neurochem.* **32**, 1047 (1979).
53. F. A. Henn and D. J. Henke, *Neuropharmacology* **17**, 985 (1978).
54. R. S. L. Chang, V. T. Tran, S. H. Snyder, *Brain Res.*, in press.
55. A. S. Lippa, M. C. Sano, J. Coupet, C. A. Klepner, B. Beer, *Life Sci.* **23**, 2213 (1978); P. Skolnick, P. J. Syapin, B. A. Paugh, S. M. Paul, *Nature (London)* **277**, 397 (1979); R. C. Speth and H. Yamamura, *Eur. J. Pharmacol.* **54**, 397 (1979).
56. W. S. Young and M. J. Kuhar, *Nature (London)*, in press.
57. C. B. Pert, M. J. Kuhar, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3729 (1976); S. F. Atweh and M. J. Kuhar, *Brain Res.* **134**, 393 (1977).
58. J. F. Tallman, J. W. Thomas, D. W. Gallager, *Nature (London)* **274**, 383 (1978).
59. G. J. Wastek, R. C. Speth, T. D. Reisine, H. I. Yamamura, *Eur. J. Pharmacol.* **50**, 445 (1978); M. S. Briley and S. Z. Langer, *ibid.* **52**, 129 (1978); I. L. Martin and J. M. Candy, *Neuropharmacology* **17**, 993 (1978).
60. M. Karobath and G. Sperk, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1004 (1979); M. Karobath, P. Placheta, M. Lippitsch, P. Krogsgaard-Larsen, *Nature (London)* **278**, 748 (1979).
61. R. F. Squires, D. I. Benson, C. Braestrup, J. Coupet, C. A. Klepner, V. Myers, B. Beer, *Pharmacol. Biochem. Behav.* **10**, 825 (1979).
62. C. Braestrup, M. Nielsen, P. Krogsgaard-Larsen, E. Falch, *Nature (London)* **280**, 331 (1979); R. F. Squires, C. A. Klepner, D. I. Benson, in *Proceedings of the First International Colloquium on Receptors*, S. Enna, M. Kuhar, G. Pepeu, Eds. (Raven, New York, in press).
63. B. Beer, C. A. Klepner, A. S. Lippa, R. F. Squires, *Pharmacol. Biochem. Behav.* **9**, 849 (1978); M. Williams and E. A. Risley, *Life Sci.* **24**, 833 (1979).
64. A. Guidotti, G. Toffano, E. Costa, *Nature (London)* **275**, 553 (1978).
65. G. Toffano, A. Guidotti, E. Costa, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4024 (1978).
66. T. Costa, D. Rodbard, C. B. Pert, *Nature (London)* **277**, 315 (1979); J. M. Candy and I. L. Martin, *ibid.* **280**, 172 (1979); D. Rodbard, T. Costa, C. B. Pert, *ibid.*, p. 73.
67. R. Pax, J. L. Bennett, N. S. Fetteker, *Naunyn Schmiedeberg's Arch Pharmacol.* **304**, 309 (1978); J. L. Bennett, *J. Parasitol.*, in press.
68. C. Braestrup and M. Nielsen, *Brain Res.* **147**, 170 (1979); J. M. Candy and I. L. Martin, *J. Neurochem.* **32**, 655 (1979).
69. D. W. Gallager, P. Mallorga, J. W. Thomas, J. F. Tallman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, in press.
70. E. J. Peck, J. M. Schaeffer, J. H. Clark, *Biochem. Biophys. Res. Commun.* **52**, 394 (1973); D. V. Greenlee and R. W. Olsen, *ibid.* **88**, 380 (1979).
71. M. Yousufi, J. W. Thomas, J. F. Tallman, *Life Sci.* **25**, 463 (1979); M. Garvish, R. S. L. Chang, S. H. Snyder, *ibid.*, in press.
72. H. Mohler, in *Proceedings of the First International Colloquium on Receptors*, S. Enna, M. Kuhar, G. Pepeu, Eds. (Raven, New York, in press).
73. L. C. Rice, A. Brossi, J. F. Tallman, S. M. Paul, P. Skolnick, *Nature (London)* **278**, 854 (1979).
74. G. Biggio, E. Costa, A. Guidotti, *Neuroscience* **2**, 49 (1977); E. Costa, A. Guidotti, G. Toffano, *Br. J. Psychiatry* **133**, 239 (1978).
75. A. Guidotti, M. Baraldi, E. Costa, *Pharmacology*, in press.
76. C. Braestrup, C. Nisson, R. F. Squires, S. Schousboe, *Neuroscience* **9**, 45 (1978).
77. J. F. Tallman, D. W. Gallager, P. Mallorga, J. W. Thomas, W. J. Strittmatter, F. Hirata, J. Axelrod, in *Proceedings of the First International Colloquium on Receptors*, S. Enna, M. Kuhar, G. Pepeu, Eds. (Raven, New York, in press).
78. A. Huang, J. L. Bosher, S. M. Paul, V. Moncada, P. Skolnick, in preparation.
79. R. S. L. Chang and S. H. Snyder, *Eur. J. Pharmacol.* **48**, 213 (1978).
80. M. J. Williamson, S. M. Paul, P. Skolnick, *Nature (London)* **275**, 551 (1978).
81. ———, *Life Sci.* **23**, 1935 (1978).
82. J. F. Tallman, J. W. Thomas, D. W. Gallager, *ibid.* **24**, 873 (1979).
83. C. B. Pert and S. H. Snyder, *ibid.* **16**, 1623 (1975); D. B. Bylund, M. E. Charness, S. H. Snyder, *J. Pharmacol. Exp. Ther.* **201**, 644 (1977).
84. B. B. Wolfe, T. K. Harden, P. B. Molinoff, *Annu. Rev. Pharmacol. Toxicol.* **17**, 575 (1977).
85. H. Mohler, T. Okada, S. J. Enna, *Brain Res.* **156**, 391 (1978).
86. H. Rosenberg and T. H. Chiu, *Life Sci.* **24**, 803 (1979); T. H. Chiu and H. Rosenberg, *ibid.* **23**, 1153 (1978); C. Braestrup, M. Nielsen, R. F. Squires, *ibid.* **24**, 347 (1979).
87. S. C. Landis, *J. Cell Biol.* **57**, 782 (1973).
88. R. C. Speth and H. I. Yamamura, *Eur. J. Pharmacol.* **54**, 397 (1979).
89. P. Rakic and R. L. Sidman, *J. Comp. Neurol.* **152**, 103 (1973).
90. H. A. Robertson, I. L. Martin, J. M. Candy, *Eur. J. Pharmacol.* **50**, 455 (1978).
91. S. M. Paul and P. Skolnick, *Science* **202**, 892 (1978).
92. P. Skolnick, L. P. Stalvey, J. W. Daly, E. Hoyler, J. N. Davis, *Eur. J. Pharmacol.* **47**, 201 (1978).
93. A. Lippa, D. Critchett, M. C. Sano, C. A. Klepner, E. N. Greenblatt, J. Coupet, B. Beer, *Pharmacol. Biochem. Behav.*, in press.
94. D. W. Gallager, P. Mallorga, J. F. Tallman, *Brain Res.*, in press.
95. S. M. Paul, P. J. Syapin, B. Paugh, V. Moncada, P. Skolnick, *Nature (London)* **281**, 688 (1979).
96. P. J. Marangos, S. M. Paul, P. Greenlaw, F. K. Goodwin, P. Skolnick, *Life Sci.* **22**, 1893 (1978).
97. M. Karobath, G. Sperk, G. Schonbeck, *Eur. J. Pharmacol.* **49**, 323 (1978).
98. P. Skolnick, P. J. Marangos, F. K. Goodwin, M. Edwards, S. M. Paul, *Life Sci.* **23**, 1473 (1978).
99. T. Asano and S. Spector, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 977 (1979).
100. P. Skolnick, P. J. Syapin, B. A. Paugh, V. Moncada, P. J. Marangos, S. M. Paul, *ibid.*, p. 1515.
101. H. Mohler, P. Polc, R. Cumin, L. Pieri, R. Kettler, *Nature (London)* **278**, 563 (1979).
102. J. F. MacDonald, J. L. Barker, S. M. Paul, P. J. Marangos, P. Skolnick, *Science* **205**, 715 (1979).
103. P. Skolnick and S. M. Paul, unpublished data.
104. A. I. Ally, M. S. Manku, D. F. Horrobin, R. A. Karmali, R. O. Morgan, M. Marmazyn, *Neuroscience* **7**, 31 (1978).
105. G. D. Colello, D. M. Hockenberry, H. B. Bosmann, S. Fuchs, K. Folkers, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 6319 (1978).
106. J. C. Nixon and J. H. Woolf, *Pharmacologist* **21**, 242 (1979).
107. T. Heidmann and J-P. Changeux, *Annu. Rev. Biochem.* **47**, 317 (1978).
108. K. V. Speeg, S. Wang, G. R. Avant, M. L. Berman, S. Schenker, *Life Sci.* **27**, 1345 (1979).
109. P. Skolnick, F. K. Goodwin, S. M. Paul, *Arch. Gen. Psychiatry* **36**, 78 (1979).