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Structure and Pharmacology of γ-Aminobutyric Acid$_A$ Receptor Subtypes

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I. Introduction

GABA is quantitatively one of the most important inhibitory transmitters in the central nervous system. It is estimated that, depending on the brain region, 20 to 50% of all central synapses use GABA as their transmitter (Bloom and Iversen, 1971; Young and Chu, 1990). The actions of GABA are mediated by at least two different receptor classes that have been defined pharmacologically: GABA$_A$ and GABA$_B$ receptors. GABA$_A$ receptors are stimulated by GABA, muscimol, and isoguvacine and are inhibited by the convulsants bicuculline (competitively) and picrotoxin (noncompetitively). These receptors are directly associated with a Cl^- ion channel (Bormann, 1988; Silvilotti and Nistri, 1991). GABA$_B$ receptors are stimulated by GABA and (-)baclofen and are inhibited by phaclofen. These receptors seem to be coupled to Ca^{2+} or K^+ channels via second-messenger systems (Bormann, 1988; Bowery, 1993). A third class of GABA receptors, the GABA$_C$ receptors, are stimulated by GABA and certain conformationally restricted analogues of GABA, such as *cis*-4-aminocrotonic acid and are insensitive to both bicuculline and (-)baclofen (Quian and Dowling, 1993; Feigenspan et al., 1993). These receptors, similar to GABA$_A$ receptors, are directly associated with a Cl^- ion channel, and recent evidence seems to indicate that GABA$_C$ receptors might be structurally related to GABA$_A$ receptors (see section III.B.6 of this article).

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†Abbreviations: GABA, γ-aminobutyric acid; propofol, 2,6-diisopropylphenol; AVM, avermectin B_1a; 4-PIOL, (5-(4-piperidyl)isoxazol-3-ol); EC_{50}, concentration of a compound producing 50% of its maximum effect; THIP, (4,5,6,7-tetrahydro-isoxazolo[4,5-c]pyridine-3-ol); TBPS, t-butylbicyclophosphorothionate; DHP, α-dihydropicrotoxinin; alphaxalone, 5α-pregnan-3α-ol-11,20-dione; DHEAS, dehydroepiandrosterone sulfate; PS, pregnenolone sulfate; loreclezole (R 72063), (2)-[2-chloro-2-(2,4-dichlorophenyl)-ethenyl]-1H-1,2,4-triazole); U 93631, (4-dimethyl-3t-butylcarboxyl-4,5-dihydro[l, 5-a]imidazoquinoxaline); SEM, standard error of the mean; EC, embryonal carcinoma; cDNA, complementary deoxyribonucleic acid; mRNA, messenger ribonucleic acid; HEK, human embryonic kidney; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; ATP, adenosine triphosphate; βCCM, methyl ester of β-carboline-3-carboxylate; βCCE, ethyl ester of β-carboline-3-carboxylate; βCCP, propyl ester of β-carboline-3-carboxylate.

II. Pharmacology of γ-Aminobutyric Acid$_A$ Receptors in Vertebrate Brain Tissue

A large body of evidence indicates that GABA$_A$ receptors are the targets of a variety of pharmacologically and clinically important drugs. Thus, binding studies and electrophysiological and behavioural experiments indicated that the anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic benzodiazepines (Study and Barker, 1981; Polc, 1988) and some depressant barbiturates (Study and Barker, 1981; Bormann, 1988) enhance the action of GABA on GABA$_A$ receptors. In contrast, some anxiogenic or convulsant β-carbolines (Bormann, 1988; Polc, 1988), the convulsants bicuculline or picrotoxinin (Olsen, 1982; Bormann, 1988), or some insecticides, such as dieldrin or lindane (Lawrence and Casida, 1984; Casida, 1993; Nagata and Narahashi, 1994) reduce the actions of GABA on this receptor. And finally, some anesthetics—such as etomidate (Olsen, 1982), propofol (Hales and Lambert, 1991), alphaxalone (Bormann, 1988), halothane and enflurane (Yang et al., 1992), and the anthelmintic avermectin B_1a (Olsen, 1982; Drexler and Sieghart, 1984a)—produce at least part of their pharmacological effects by interacting with GABA$_A$ receptors. The chemical structure of some of these compounds is shown in figures 1, 2, and 3.

A large series of experiments indicated that, in most cases, the above-mentioned compounds do not interact directly with the GABA binding site but exert their action by binding to additional allosteric sites at GABA$_A$ receptors. This binding induces a conformational change in the GABA$_A$ receptors that in turn influences the binding properties of other binding sites present on these

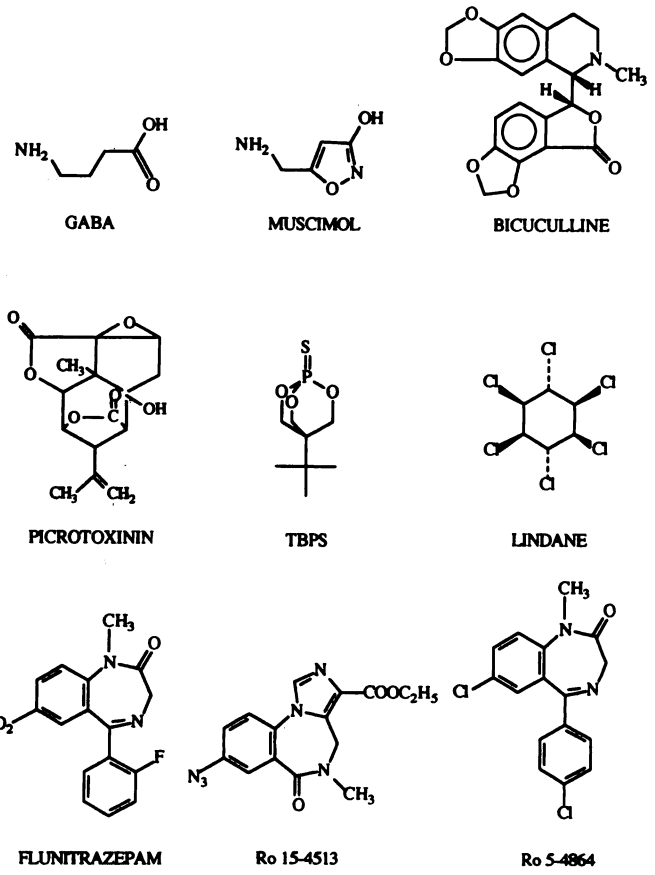


FIG. 1. Ligands for the GABA, picrotoxinin, or benzodiazepine binding site.

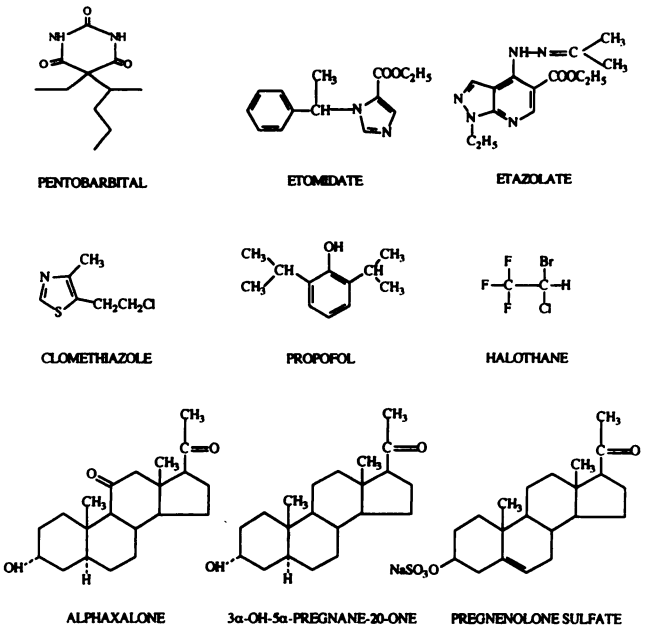


FIG. 2. Ligands for the barbiturate, anesthetic, or steroid binding site.

receptors (causing complex allosteric interactions of these binding sites) and modulates GABA-induced chloride ion flux (Sieghart, 1992).

The basic information on the pharmacology of GABA$_A$ receptors has been derived from studies using either intact brain, brain slices, or brain membranes. Recent recombinant receptor studies have supported and extended our knowledge on these receptors and provided final evidence for the existence of a multiplicity of GABA$_A$ receptors in the brain. In order to set the stage for the discussion of the pharmacology of individual GABA$_A$ receptor subtypes, the average properties of GABA$_A$ receptors, and the interaction of their multiple binding sites as characterized in studies using vertebrate brain tissue will be summarized first. The properties of invertebrate GABA$_A$ receptors seem to be different from those of vertebrate receptors and will not be discussed in this article. The interested reader is referred to recent review articles on these receptors (Rauh et al., 1990; Darlison, 1992; Casida, 1993).

A. The γ-Aminobutyric Acid Binding Sites of γ-Aminobutyric Acid$_A$ Receptors

GABA, by binding to GABA$_A$ receptors, increases the neuronal membrane conductance for Cl^- ions. Because the chloride ion concentration within neuronal cells is low in most cases, the chloride gradient across the membrane forces Cl^- into the cell. At the resting membrane potential, however, this effect is more or less balanced by the electrochemical driving force that inhibits Cl^- entry because of the negative charge inside of the cell. Opening of chloride ion channels in unexcited neurons thus usually results in a slight membrane hyperpolarization and in a reduced neuronal excitability of the cells, because the increased chloride ion conductance counteracts the effects of depolarizing stimuli (Study and Barker, 1981; Bormann, 1988). In some cases, however, especially in developing brain tissue (Cherubini et al., 1991), but also in some neurons from adult brain (Avoli, 1992) as well as in astrocytes and oligodendrocytes (Von Blankenfeld and Kettenmann, 1991), GABA has been demonstrated to produce excitatory actions. This seems to be because of an increased chloride ion concentration

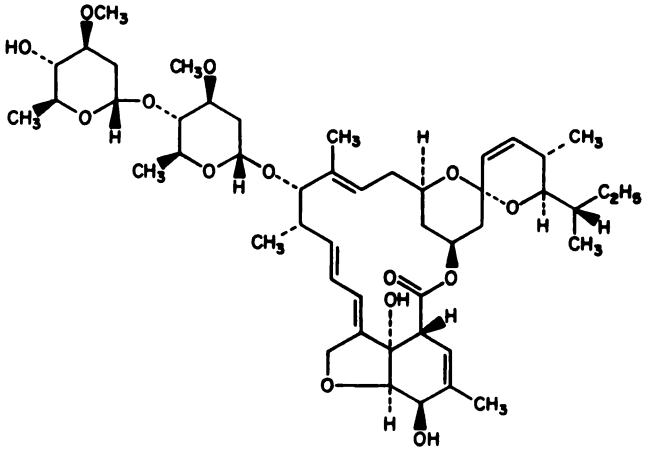


FIG. 3. Avermectin B_1a.

in these cells. The chloride gradient across the membrane of the respective cells is thus smaller than in cells where GABA exerts a hyperpolarizing action. On opening of chloride ion channels, the electrochemical driving force pushes Cl^- ions out of the cells and this results in a depolarization of the membrane potential.

The inhibitory as well as the excitatory effect of GABA can be blocked by bicuculline or picrotoxin (fig. 1) (Cherubini et al., 1991; Von Blankenfeld and Kettenmann, 1991; Avoli, 1992; Macdonald and Twyman, 1992), indicating that in each case, a $GABA_A$ receptor is involved. In addition to bicuculline, several other compounds, such as the steroid R 5135 (Hunt and Clements-Jewery, 1981) or the arylaminopyridazines SR 95103 (Chambon et al., 1985) and SR 95531 (Heaulme et al., 1987), competitively inhibit GABA-induced chloride ion flux. Most of the other GABA antagonists so far described have a lower potency than bicuculline for the inhibition of GABA-induced effects in the brain (Dalkara et al., 1986). Recently, however, several muscimol and thiomuscimol derivatives with potent GABA-antagonistic properties have been synthesized (Melikian et al., 1992), which now have to be further characterized.

The GABA binding site on the $GABA_A$ receptor can be selectively labeled by agonists such as [^{3}H]GABA or [^{3}H]muscimol (fig. 1) when endogenous GABA is removed from brain membranes by multiple washing steps and when sodium-free buffer and low temperatures are used to inactivate the GABA-transport system (Olsen, 1982; Schumacher and McEwen, 1989). This site shows both high and low affinity for GABA and its agonists with K_D values in the low and high nanomolar range, respectively (Olsen et al., 1981). This heterogeneity of binding sites is observed whether the assay is performed at 0°C or at higher temperature (22 or 37°C) and seems not to depend on the brain region or mammalian species investigated (Olsen et al., 1984). The low affinity $GABA_A$ recognition site seems to be an antagonist-preferring site, because it can be selectively labeled by specific antagonists such as (+)bicuculline (Olsen and Snowman, 1983) or SR 95531 (Heaulme et al., 1987). Both the low and high affinity forms of the $GABA_A$ binding site show similar drug specificity (Olsen et al., 1984) and are immunologically similar (De Blas et al., 1988). In addition, pentobarbital increases the number of high affinity sites at the expense of low affinity sites (Yang and Olsen, 1987). Thus, although a separate existence of $GABA_A$ receptors exhibiting either high or low affinity GABA binding sites cannot be excluded, at least some of these sites presumably represent different conformational states of the same receptor.

It is now generally assumed that GABA exerts its physiological effects by acting at very low affinity binding sites. Thus, micromolar concentrations of GABA or its analogues are necessary to activate chloride ion channels in electrophysiological (Segal and Barker, 1984) and ion flux experiments (Cash and Subbarao, 1987a, b; Kardos and Cash, 1990) and to modulate other binding sites at the $GABA_A$ receptor (Karobath et al., 1979; Olsen, 1982; Squires et al., 1983). Such concentrations are higher than those that the low affinity [^{3}H]GABA binding sites would require to be fully activated. This and the apparent increase in the number of high and low affinity binding sites on allosteric modulation of [^{3}H]GABA binding (Olsen and Snowman, 1982, 1983; Corda et al., 1986b, 1987) (see next page) are the reasons that the additional existence of "very low affinity" GABA binding sites is assumed.

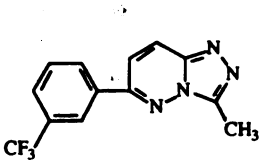
These data indicating the separate existence of high, low, and very low affinity binding sites that partially can be interconverted into each other can be explained if it is assumed that there are several distinct GABA binding sites on a single $GABA_A$ receptor. Molecular biological evidence discussed in III.B.2 of this article indicates that up to five GABA binding sites might be present on a single $GABA_A$ receptor. These GABA binding sites in the unoccupied state might have a similar high affinity for GABA agonists. On increasing occupation of these sites with GABA, the affinity of the remaining unoccupied sites might allosterically become reduced. The high and possibly the low affinity GABA sites probably are constantly occupied under the physiological GABA concentration present in the synaptic cleft. Occupation of these sites does not cause an opening of chloride channels and can only adequately be measured by binding studies when most of the GABA is removed by extensively washing of the membranes.

Under physiological conditions, probably only two or three GABA binding sites with affinities of about 100 μM are unoccupied per $GABA_A$ receptor. This can be concluded from $^{36}Cl^-$ transmembrane flux measurements using quench flow techniques (Cash and Subbarao, 1987a, b; Kardos and Cash, 1990) with reaction times that allow the resolution of receptor desensitization rates from the ion flux rates. These investigations were supported by electrophysiological studies indicating that there might be three open states of the channel, and that most $GABA_A$ receptor channels open after the binding of more than one GABA molecule (Bormann, 1988; Macdonald and Twyman, 1992). And finally, recent studies that examined $GABA_A$ receptor activation, following rapid applications of GABA to outside-out patches excised from cultured postnatal rat cerebellar neurons, indicated that the final GABA binding step was of extremely low affinity (about 500 μm); it was estimated that the cleft concentration of GABA reached at least 500 μM (Maconochie et al., 1994). The very low affinity of the remaining unoccupied sites ensures that GABA-activated chloride channels can only be opened under conditions of synaptic transmission where GABA is massively released into the synaptic cleft.

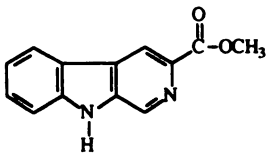
The $GABA_A$ agonists muscimol and isoguvacine, were able to activate chloride conductance and to allosterically modulate binding of ligands to other binding sites

at GABA$_A$ receptors (see section II.B. and II.C) to an extent similar to that of GABA. These compounds, thus, seem to act as full GABA$_A$ agonists. Other compounds, such as 4-PIOL, THIP, or piperidine 4-sulphonic acid, exhibited weaker actions at the GABA binding site (Olsen, 1982; Kristiansen et al., 1991). Thus, in electrophysiological experiments 4-PIOL activated chloride ion channels in a manner similar to that of isoguvacine, and these actions were blocked by the GABA$_A$ antagonist bicuculline. 4-PIOL, however, although with a weaker potency than bicuculline (Kristiansen et al., 1991), antagonized the response to isoguvacine with a parallel shift to the right of the dose-response curve. In addition, 4-PIOL, as well as THIP, taurine, piperidine-4-sulphonic acid and 3-aminopropane-sulphonic acid, exhibited only a weak or no effect (Falch et al., 1985) on benzodiazepine binding (see section II.B.), but did antagonize muscimol-stimulated benzodiazepine binding to rat cortical membranes (Falch et al., 1990). These compounds thus exhibit partial agonist actions at the GABA$_A$ binding site.

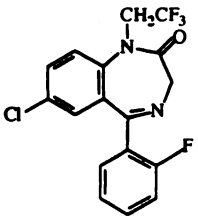
In agreement with the notion that GABA$_A$ receptors contain a multiplicity of allosteric binding sites, GABA binding to these receptors can be modulated by a variety of different compounds. Thus, [^{3}H]GABA or [^{3}H]muscimol binding to the GABA binding site of GABA$_A$ receptors could be enhanced by benzodiazepines (Skerritt et al., 1982a, b; Korneyev, 1983; Corda et al., 1986b; Bristow et al., 1990) and by Cl 218872 (fig. 4) and zopiclone, two nonbenzodiazepine ligands of the benzodiazepine binding site of GABA$_A$ receptors (Skerritt and Johnston, 1983a); this effect could be antagonized by the benzodiazepine antagonist Ro15–1788 (Skerritt and Johnston, 1983a; Korneyev, 1983). Whereas some reports indicated that agonist benzodiazepines enhanced [^{3}H]GABA binding by increasing the affinity of the "low affinity"



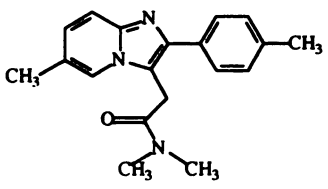
Cl 218872



β-CCM



2-OXOQUAZEPAM



ZOLPIDEM

FIG. 4. Type I benzodiazepine receptor-selective ligands.

GABA$_A$ receptor (Skerritt et al., 1982a; Skerritt and Johnston, 1983a; Bristow et al., 1990), other studies suggested that the effect was caused by an increase in the number of high and low affinity GABA$_A$ receptors (Corda et al., 1986b, 1987).

Because very low affinity GABA binding sites are involved in opening of chloride ion channels, it likely that benzodiazepines, if they modulate GABA or muscimol binding at all (see next paragraph), will modulate the very low affinity GABA binding. This modulation will not necessarily show up at the high and medium affinity GABA or muscimol binding sites that can be investigated using binding studies, and the results obtained might vary with the degree of occupation of these sites by GABA molecules. This conclusion is in agreement with the observation that benzodiazepine stimulation of GABA binding is not easily observed and is sensitive to membrane manipulations and assay conditions (Skerritt et al., 1982a). The large concentration range and the high final concentration of radioactively labeled ligand that had to be used in these experiments might have caused additional problems with an exact determination of kinetic constants.

However, electrophysiological experiments indicated that benzodiazepine enhancement of GABA$_A$ receptor current cannot be purely caused by an increased affinity of the receptor for GABA (for review see Macdonald and Twyman, 1992). In addition, it was demonstrated (Edgar and Schwartz, 1992) that [^{3}H]muscimol, under conditions used in $^{36}Cl^-$ uptake assays (a measure of receptor function), bound to a population of receptors with a K_D (2 μM) similar to its EC_{50} value for $^{36}Cl^-$ uptake. Under these conditions, the benzodiazepine diazepam enhanced the potency of muscimol in ion flux experiments (without changing the maximal ion flux), but did not alter the number or affinity of [^{3}H]muscimol binding sites. From these results the authors concluded that benzodiazepines enhance GABAergic function by increasing receptor-ion channel coupling, rather than by increasing GABA$_A$ receptor affinity (Edgar and Schwartz, 1992). It is thus possible that benzodiazepines, by increasing the efficiency of GABA to open its chloride channel, cause an enhanced frequency of GABA-induced chloride channel openings, an effect that is observed in electrophysiological experiments (Study and Barker, 1981). This might enable a single GABA molecule to open chloride ion channels and thus enhance GABA-induced chloride channel opening already at lower GABA concentrations. A recent report indicating that a single GABA molecule is able to open chloride channels in the presence but not in the absence of chlordiazepoxide (Serfozo and Cash, 1992) supports this conclusion.

In addition to benzodiazepine agonists, other compounds allosterically interacting with the GABA$_A$ receptor modulate [^{3}H]GABA or [^{3}H]muscimol binding. For example, pentobarbital, etazolate, and etomidate (fig. 2),

enhanced [^{3}H]GABA or [^{3}H]muscimol binding (Placheta and Karobath, 1980; Olsen and Snowman, 1982; Skerritt and Johnston, 1983b; Borea et al., 1983), and this effect could be modulated by benzodiazepine receptor ligands (Borea et al., 1983), indicating a simultaneous allosteric interaction of the GABA-, the barbiturate- and the benzodiazepine binding site. Similarly, pregnanolone was able to stimulate [^{3}H]muscimol binding, and this effect could be inhibited by picrotoxinin (Kirkness and Turner, 1988). Picrotoxinin, on the other hand, allosterically and only partially inhibited binding of [^{3}H]GABA or [^{3}H]muscimol to the GABA binding site of $GABA_A$ receptors (Skerritt and Johnston, 1983b).

B. The Benzodiazepine Binding Sites of γ-Aminobutyric Acid$_A$ Receptors

Electrophysiological experiments in many different neuronal systems have indicated that benzodiazepines, such as diazepam or flunitrazepam (fig. 1), enhance the actions of GABA at the $GABA_A$ receptor by increasing the frequency of Cl^- channel opening with little effect on the channel open time or channel conductance (Study and Barker, 1981). Single channel analysis indicated that this increase in channel opening frequency is not due to single channel events but to an increased occurrence of bursting activity while burst durations are not altered (Macdonald and Twyman, 1992; Macdonald and Olsen, 1994). Benzodiazepines are inactive, however, at the chloride ionophore in the absence of GABA (Study and Barker, 1981; Polc, 1988).

Biochemical experiments demonstrated the existence of specific high affinity binding sites for benzodiazepines on brain membranes that are closely associated with $GABA_A$ receptors (Braestrup and Squires, 1977; Möhler and Okada, 1977). Thus, binding of [^{3}H]flunitrazepam to brain membranes was chloride-dependent and stimulated by GABA or muscimol (Tallman et al., 1978; Karobath and Sperk, 1979; Olsen, 1982) and a large variety of GABA analogues (Braestrup and Nielsen, 1983), and this stimulation was inhibited by (+)bicuculline and other $GABA_A$ receptor antagonists. In these experiments, GABA and $GABA_A$ agonists increased the affinity of the benzodiazepine receptors for benzodiazepines without changing the number of binding sites (Olsen, 1982). In addition, binding of benzodiazepines to this high affinity benzodiazepine binding site was stimulated by some depressant barbiturates (such as pentobarbital or secobarbital), by some anesthetics (such as etomidate or alphaxalone), some anxiolytic, anticonvulsant and hypnotic steroids, and by the anthelmintic and insecticidal AVM (Supavilai and Karobath, 1981b; Olsen, 1982; Thyagarajan et al., 1983; Harrison and Simmonds, 1984; Gee, 1988).

Other studies have indicated that benzodiazepines not only influenced the binding of GABA to $GABA_A$ receptors (see section II.A.), but also modulated the high affinity binding of TBPS (fig. 1) (Supavilai and Karobath, 1983) that is thought to be closely associated with the Cl^- ion channel of the $GABA_A$ receptor (see below).

Because there was an excellent correlation between the clinical potency of benzodiazepines and their affinity for the [^{3}H]flunitrazepam binding site, it is now believed that these $GABA_A$ receptor-associated binding sites ("central" benzodiazepine receptors) are the pharmacological receptors by which the benzodiazepines exert their clinically important actions (Haefely et al., 1985). Other benzodiazepine binding sites, the "peripheral" benzodiazepine binding sites (Verma and Snyder, 1989) localized on the outer mitochondrial membrane of many tissues including brain, or the "micromolar" benzodiazepine binding sites (Bowling and DeLorenzo, 1982) are pharmacologically distinct from and unrelated to these $GABA_A$ receptor associated benzodiazepine binding sites. Because there is no significant correlation between the clinical potency of benzodiazepines and their affinity for the "peripheral" or "micromolar" benzodiazepine binding sites, these sites are probably not involved in most of the clinical actions of benzodiazepines.

In a search for compounds with a more selective action than that of the classical benzodiazepines, many ligands with a benzodiazepine or nonbenzodiazepine structure were identified that exhibited a high affinity for the $GABA_A$ receptor-associated benzodiazepine binding site (Braestrup and Nielsen, 1983; Haefely et al., 1985; Gardner et al., 1993). Some of these ligands, the "benzodiazepine receptor agonists" like the classical benzodiazepines enhanced GABA-induced chloride ion flux (positive intrinsic efficacy). These compounds have anxiolytic, anticonvulsant, muscle relaxant, and sedative hypnotic properties. Other ligands, the "inverse benzodiazepine receptor agonists", reduced GABA-induced chloride flux (negative intrinsic efficacy) by a mechanism opposite to the action of benzodiazepine receptor agonists (Macdonald and Twyman, 1992). These compounds have convulsant, stimulant, and anxiogenic effects (Polc et al., 1982; Braestrup et al., 1982). A third group of high affinity ligands, the "benzodiazepine receptor antagonists," had no or only a weak intrinsic efficacy for changing the GABAergic transmission. These compounds therefore have no or only weak effects when given to animals or humans, but are able to inhibit the effects of both benzodiazepine receptor agonists or inverse benzodiazepine receptor agonists (Polc et al., 1982; Braestrup et al., 1982). Between these extreme actions, compounds were identified (partial agonists or partial inverse agonists) with intermediate actions. Such compounds have less positive or negative intrinsic efficacy than full agonists or inverse agonists (Braestrup et al., 1984; Haefely et al., 1985).

The agonist, inverse agonist, or antagonist property of benzodiazepine receptor ligands was investigated by exploiting a variety of allosteric interactions between the benzodiazepine binding site and other binding sites at the $GABA_A$ receptor (Braestrup et al., 1984). Thus,

GABA ("GABA shift," Möhler and Richards, 1981; Braestrup et al., 1982), barbiturates ("barbiturate shift," Honore et al., 1984), or etazolate and etomidate ("etazolate or etomidate shift," Ehlert et al., 1982) enhance the potency of benzodiazepine receptor agonists and reduce the potency of inverse benzodiazepine receptor agonists for displacement of radiolabeled benzodiazepine receptor ligands. The potency of antagonists, however, is not influenced by these allosteric modulators of GABA$_A$ receptors. Similarly, benzodiazepine receptor agonists enhance, and inverse benzodiazepine receptor agonists reduce, [^{35}S]TBPS binding, whereas benzodiazepine receptor antagonists are ineffective in this binding assay ("TBPS shift") (Supavilai and Karobath, 1983; Braestrup et al., 1984). Other studies have indicated that the effects of benzodiazepine binding site ligands on [^{35}S]TBPS binding were concentration-dependent. Thus, partial agonists or partial inverse agonists reached the same degree of modulation of [^{35}S]TBPS binding at higher receptor occupancies than full agonists or inverse agonists (Maksay, 1993).

An additional method that distinguishes between benzodiazepine agonists, antagonists, and inverse agonists utilizes the changes in the affinity of these compounds for benzodiazepine binding sites induced by a partial, irreversible labeling of GABA$_A$ receptor-associated benzodiazepine binding sites ("photoshift," Karobath and Supavilai, 1982; Braestrup et al., 1984). For example, [^{3}H]flunitrazepam (Möhler et al., 1980), can be used as a photoaffinity label for the benzodiazepine binding site of GABA$_A$ receptors (see also section IV. B. of this article). But even under optimal conditions, specific irreversible binding of flunitrazepam to brain membranes occurred to only about 25% of the available [^{3}H]flunitrazepam binding sites. The remaining binding sites, however, seemed to change their affinity for benzodiazepine receptor agonists about 20- to 100-fold, whereas their affinity for benzodiazepine receptor antagonists or inverse benzodiazepine receptor agonists was unchanged or even slightly increased, respectively (Karobath and Supavilai, 1982). This observation is supported by an experiment indicating that for every flunitrazepam molecule specifically and irreversibly bound to membranes during irradiation with ultraviolet light, about three molecules of [^{3}H]flunitrazepam dissociated from previously occupied benzodiazepine binding sites (Sieghart and Drexler, 1983). Similar results were obtained when this experiment was performed in cerebellum, hippocampus, and cerebral cortex. In a complimentary experiment it was demonstrated that the partial inverse benzodiazepine receptor agonist [^{3}H]Ro15–4513 could be used as a photoaffinity label for GABA$_A$ receptors and that this compound, in contrast to [^{3}H]flunitrazepam, was able to label 100% of the available benzodiazepine binding sites (Sieghart et al., 1987).

These data can be explained if the existence of several benzodiazepine binding sites on GABA$_A$ receptors is assumed. Each of these binding sites probably can assume at least two different conformations: one exhibiting a high and one a low affinity for benzodiazepine receptor agonists (Monod et al., 1965). In the undisturbed receptors, these conformations presumably are freely interconvertible. [^{3}H]flunitrazepam reversibly binds to the high affinity conformation and by shifting the equilibrium is able to occupy most of the binding sites. Photolabeling of the receptor by [^{3}H]flunitrazepam, however, inhibits the free conformational change in the photolabeled receptor and favors the low affinity conformation in the other benzodiazepine binding sites on the same receptor. This causes dissociation of reversibly bound [^{3}H]flunitrazepam from these sites. The benzodiazepine antagonist Ro15–1788, and the partial inverse benzodiazepine agonist Ro15–4513 can interact with both conformations to a similar extent. These compounds, thus, are able to label the remaining binding sites.

Similarly, it is possible that reversible binding of a benzodiazepine receptor agonist, antagonist, or inverse agonist to one of the several benzodiazepine binding sites on GABA$_A$ receptors could allosterically change the conformation and thus the affinity of the other sites for benzodiazepine receptor ligands. This assumption is supported by experiments suggesting cooperative interactions between benzodiazepine binding sites of GABA$_A$ receptors. It was demonstrated that the rate of dissociation of [^{3}H]flunitrazepam from its binding sites in brain membranes is accelerated by the occupation of unlabeled binding sites by diazepam, flunitrazepam, or other benzodiazepine binding site ligands (Doble, 1982; Chiu and Rosenberg, 1985). A cooperative interaction between several benzodiazepine binding sites present in a single GABA$_A$ receptor could be one explanation for the observation that treatment with benzodiazepine receptor agonists seems to sensitize GABA$_A$ receptors for inverse benzodiazepine receptor agonists (Nutt et al., 1992). It has to be stressed, however, that an acceleration of dissociation not necessarily is due to an allosteric interaction of binding sites, but could also be due to a competition for individual attachment points on the same binding site (Prinz and Striessnig, 1993).

Although there is a rough agreement between these different methods in the agonist-to-inverse agonist ranking of the various compounds investigated, depending on the conditions used, discrepancies between the in vivo action of compounds and their apparent in vitro agonistic or inverse agonistic activities do occur (Braestrup et al., 1984; Honore et al., 1984; Dawson and Poretski, 1989), possibly reflecting additional factors such as receptor heterogeneity or ability of the individual compounds to cause conformational changes in the GABA$_A$ receptor complex at low temperatures (0–4°C) where most of the measurements were performed (Dawson and Poretski, 1989). In addition, it is possible that the extent of the agonistic or inverse agonistic effects of benzodiazepine binding site ligands depends on the con-

formational state of the $GABA_A$ receptor-chloride ionophore complex. It has been demonstrated that the benzodiazepine binding site antagonist Ro15–1788 did not alter the effect of 30 μM GABA on $^{36}Cl^-$ uptake. However, it did inhibit the $^{36}Cl^-$ uptake produced by 100 μM GABA and enhanced $^{36}Cl^-$ uptake mediated by 10 μM GABA (Malatynska et al., 1991). The effect of this compound on GABA-induced Cl^- flux, thus, changes with the extent of $GABA_A$ receptor activation. It is quite possible that the effects of not only the benzodiazepine site ligands, but also those of other allosteric ligands of $GABA_A$ receptors, depend on the overall state of activation of these receptors.

Recently, it has been demonstrated that some benzodiazepine binding site ligands, such as β-carboline-3-carboxylate esters (Chiu and Rosenberg, 1985; Dellouve-Courillon et al., 1989) or the anxiolytic cyclopyrrolones zopiclone and suriclone (Trifiletti and Snyder, 1984), don't interact in a purely competitive way with these binding sites. Although it might be concluded that these compounds bind to regions or domains of this site that are different from those interacting with benzodiazepines, in the light of the arguments discussed above the possibility cannot be excluded that these compounds competitively interact with one of several benzodiazepine binding sites on $GABA_A$ receptors and by this cause an allosteric change in the ligand affinity of the remaining binding sites.

C. The Picrotoxinin/TBPS Binding Sites of γ-Aminobutyric Acid$_A$ Receptors

Picrotoxinin, pentylenetetrazol, some bicyclic cage compounds, and a variety of insecticides are convulsants that antagonize GABA-induced chloride conductance responses (Olsen, 1982; Bormann, 1988; Casida, 1993; Nagata and Narahashi, 1994) (fig. 1). The majority of the electrophysiological experiments have been carried out with picrotoxin, an equimolar mixture of the inactive picrotin, and the active compound picrotoxinin (Yoon et al., 1993), because picrotoxinin exhibits a rapid onset of activity (milliseconds). This is in contrast to, for instance, the cage convulsant TBPS, which has a slow onset of action and needs about 30 min to reach peak effect (Yoon et al., 1993). Both the onset and the recovery from GABA current blockade produced by picrotoxinin and similar convulsants depend on the presence of GABA or $GABA_A$ agonists. It was therefore concluded that the mechanism of picrotoxinin blockade of the GABA-Cl^- ionophore requires an open channel and that the picrotoxinin binding site is located within the channel (Inoue and Akaike, 1988; Inomata et al., 1988). Recent experiments, however, seem to question this conclusion (Yoon et al., 1993).

Picrotoxinin and cage convulsants only partially and allosterically inhibited GABA receptor binding (Skerrit and Johnston, 1983b) and did not displace benzodiazepines from their high affinity binding sites (Olsen, 1982), but allosterically modulated benzodiazepine receptor binding (Karobath et al., 1981). Binding sites identified by [^{3}H]DHP (Olsen, 1982) or the cage convulsant [^{35}S]TBPS (fig. 1), which exhibits a high affinity for the picrotoxinin binding site and a better signal-to-noise ratio than [^{3}H]DHP (Squires et al., 1983), are strongly modulated by halide ions and thus seem to be closely associated with the chloride ion channel of the $GABA_A$ receptor. Convulsant compounds, such as picrotoxinin, pentylenetetrazole or the convulsant barbiturate isomer S(+)MPPB (S(+)N-methyl-5-phenyl-5-propylbarbituric acid), that bind to the DHP/TBPS site, induced a monophasic dissociation of [^{35}S]TBPS from its binding sites, and the rate of dissociation was identical when initiated by any of these convulsants, suggesting that these convulsants bind competitively to TBPS sites (Maksay and Ticku, 1985b).

GABA, at micromolar concentrations, allosterically inhibited [^{35}S]TBPS binding, and this effect could be reversed by $GABA_A$ receptor blockers (Maksay and Ticku, 1985a; Maksay and Simonyi, 1986; Squires and Saederup, 1987). Similarly, compounds that mimic or facilitate the effects of GABA on the $GABA_A$ receptor (e.g., barbiturates, etazolate, etomidate, and steroids, see sections II.D. and II.E.), allosterically inhibited [^{35}S]TBPS binding by reducing its binding affinity (Gee, 1988), and these effects are modulated in the presence of GABA (Im and Blakeman, 1991). In contrast, the GABA facilitating benzodiazepines inhibited the binding of [^{35}S]TBPS only in the presence of micromolar quantities of GABA (Gee, 1988). Upon abolishment of GABA action by the use of bicuculline these compounds stimulated [^{35}S]TBPS binding (Im and Blakeman, 1991) as mentioned above ("TBPS shift") (see section II.B.) (Supavilai and Karobath, 1983; Braestrup et al., 1984). The latter finding coincides with the observation that benzodiazepines affect membrane conductance to chloride ions only in the presence of GABA.

Compounds reducing the efficacy of GABA at $GABA_A$ receptors, such as some convulsant β-carbolines, in the absence as well as in the presence of GABA enhanced [^{35}S]TBPS binding affinity through specific interactions with the benzodiazepine receptor (Im and Blakeman, 1991). Thus, the high affinity TBPS binding might be associated with the "closed" conformation of the chloride ion channel (Gee, 1988). In addition, it appears that the degree of [^{35}S]TBPS binding in the presence of GABA closely reflects the functional state of $GABA_A$ receptors and may be useful for characterization of allosteric interactions between various sites on these receptors (Im and Blakeman, 1991).

Interestingly, in most studies investigating the allosteric modulation of [^{35}S]TBPS binding, biphasic effects were observed. Thus, many compounds stimulated TBPS binding at low and inhibited this binding at higher concentrations. These effects, at least partially, can be explained by the use of nonequilibrium conditions

in these binding assays (Maksay and Simonyi, 1986, 1988; for a detailed discussion see section II. N.). Thus, care has to be taken to be in binding equilibrium (180 min incubation at room temperature) when the effects of various GABA$_A$ receptor ligands on [^{35}S]TBPS binding are investigated. Alternatively, nonequilibrium conditions could be used deliberately to investigate the interaction of GABA$_A$ receptor ligands with [^{35}S]TBPS binding sites (Maksay and Simonyi, 1986, 1988). The kinetic modulation of the convulsant TBPS binding by agonists and antagonists of benzodiazepine receptors possibly could offer a suitable in vitro system to characterize not only the efficacy but also the potency of these agents for modulation of the GABA$_A$ receptor chloride ionophore complex.

D. The Interaction of Barbiturates with γ-Aminobutyric Acid$_A$ Receptors

Sedative hypnotic barbiturates, such as pentobarbital (fig. 2), phenobarbital, or secobarbital, in electrophysiological studies enhanced the actions of GABA by increasing the average channel open duration but did not alter receptor conductance or opening frequency (Study and Barker, 1981; Macdonald and Twyman, 1992; Macdonald and Olsen, 1994). At higher concentrations (> 50 μM), which are reached in plasma during anesthesia with pentobarbital (Franks and Lieb, 1994), barbiturates are able to directly open GABA$_A$ receptor-associated chloride channels in the absence of GABA (Bormann, 1988; Inomata et al., 1988). These distinct effects of barbiturates indicate the existence of at least two sites of interaction of barbiturates with GABA$_A$ receptors. Additional barbiturate binding sites on the same receptor might be involved in the modulation of desensitization of GABA$_A$ receptors (Cash and Subbarao, 1988; see section V.A. of this article).

Binding of barbiturates to GABA$_A$ receptors could not be investigated directly because of the low affinity of these compounds for these receptors. However, information on the interaction of barbiturates with GABA$_A$ receptors could be obtained by investigating other binding sites at these receptors. Barbiturates enhanced the affinity of the [^{3}H]GABA, [^{3}H]muscimol, or [^{3}H]flunitrazepam binding sites for their respective ligands in a chloride-dependent way and in a manner that correlated with their order of potency as anesthetics and hypnotics (Olsen, 1982). In addition, barbiturates inhibited the binding of [^{3}H]DHP or [^{35}S]TBPS again in the rank order of potency as hypnotics (Olsen, 1982; Squires et al., 1983). Whereas convulsants such as picrotoxinin, TBPS, pentamethylenetetrazole, and some convulsant barbiturates inhibited TBPS binding competitively, depressant barbiturates (pentobarbital, secobarbital) and related compounds, such as etazolate and etomidate (fig. 2), seemed to allosterically interact with the [^{35}S]TBPS binding sites (Supavilai and Karobath, 1984; Maksay and Ticku, 1985a, b; Ticku and Rastogi, 1986). This seems to indicate that the depressant barbiturates, as well as etazolate and etomidate, enhance GABA-induced chloride ion flux by interacting with binding sites that are different from those for GABA agonists, for benzodiazepines, or for DHP/TBPS. As with the GABA or benzodiazepine site, partial agonists seem to exist for the barbiturate binding sites (Olsen, 1982).

E. The Interaction of Steroids with γ-Aminobutyric Acid$_A$ Receptors

Several steroids, such as the anesthetic alphaxalone or the sedative hypnotic, anxiolytic, and anticonvulsant 3α-hydroxylated, 5α- or 5β- reduced metabolites of progesterone and deoxycorticosterone (fig. 2) at low concentrations (30 to 300 nM) enhance GABA-stimulated chloride conductance (Majewska, 1992; Kokate et al., 1994). At higher (> 1 μM) concentrations, which occur during surgical anesthesia with alphaxalone in humans (Cottrell et al., 1987), these compounds, like barbiturates, produce a direct opening of the GABA$_A$ receptor-associated Cl^- channel that could be inhibited by the GABA$_A$ receptor antagonist bicuculline (Callachan et al., 1987; Majewska, 1992). As with barbiturates, this points to an interaction of steroids with at least two different sites at GABA$_A$ receptors. Recently, a benz[e]indene compound, a tricyclic molecule that can be envisioned as a steroid without an A-ring, has been identified that reversibly potentiated GABA currents, presumably by interacting with the steroid binding site of GABA$_A$ receptors. This compound, however, in contrast to steroids active at the GABA$_A$ receptors, did not directly activate a membrane current and might thus be useful for determining the mechanism by which steroids potentiate GABA responses and in which way this mechanism is different from that which enables steroids to directly gate chloride channels (Rodgers-Neame et al., 1992). Steroids active at the GABA$_A$ receptor increased both the frequency (i.e., a benzodiazepine-like effect) and duration (i.e., barbiturate-like effect) of chloride channel opening (Peters et al., 1988).

In addition, these compounds facilitated GABA-stimulated uptake of $^{36}Cl^-$ by rat brain synaptoneurosomes (Harrison et al., 1987) and enhanced the binding of the GABA$_A$ agonist [^{3}H]muscimol. This enhancement seemed to be caused by an increase in binding affinity (Harrison et al., 1987) or to an increase in the number of binding sites (Lopez-Colome et al., 1990). Other experiments indicated that these compounds enhanced the affinity of the benzodiazepine receptor agonist [^{3}H]flunitrazepam in a picrotoxin-sensitive way and allosterically inhibited binding of [^{35}S]TBPS to the receptor (for review see Gee, 1988; Schumacher and McEwen, 1989; Majewska, 1992). In addition, preliminary evidence indicates that these steroids allosterically interact with the Ro5–4864 binding site (see section II.G.) of the GABA$_A$ receptor (Belelli et al., 1990).

Other experiments indicated that barbiturates potentiated steroid-activated transmembrane currents (Peters et al., 1988). In addition, barbiturates, in studies investigating [^{35}S]TBPS or [^{3}H]flunitrazepam binding, interacted with steroids in a manner inconsistent with competition at a common site (for review see Gee, 1988). These experiments provided evidence for a site of action of steroids distinct from the binding sites for GABA, benzodiazepines, barbiturates, and DHP/TBPS.

In addition to steroids that enhance the actions of GABA on $GABA_A$ receptors, other steroids, such as PS (fig. 2) and DHEAS, have been identified that act as noncompetitive antagonists at this receptor (Majewska, 1992). These compounds inhibit GABA-induced currents in a noncompetitive manner and exhibit excitatory actions on neurons. Although both PS and DHEAS inhibit GABA-induced currents, there are differences between these steroids in their mode of actions (Majewska, 1992). Whereas DHEAS acts primarily as an antagonist at $GABA_A$ receptors, PS exhibits mixed GABA-agonistic/antagonistic features.

Recently, [^{3}H]PS and [^{3}H]DHEAS have been used in binding studies. These two steroids seem to bind specifically to at least two populations of binding sites in crude synaptosomal membranes from rat brain. Although binding of [^{3}H]PS could be inhibited by DHEAS and binding of [^{3}H]DHEAS could be inhibited by PS, their specific sites of binding seem distinct. In addition, the relationship of these binding sites to the $GABA_A$ receptor so far seems unclear (Majewska, 1992).

The highly lipophilic nature of the steroids and the evidence that phospholipids are capable of binding steroids with high specificity raise the possibility that the effects of steroids are mediated by specific interactions with the membrane-lipid $GABA_A$ receptor protein interface (Gee, 1988). This possibility seems to be supported by recent results on the sensitivity of [^{3}H]PS or [^{3}H]DHEAS binding sites to protein-destructive treatment or to treatment with phospholipase A_2 (Majewska, 1992). The site of action of steroids, however, seems to be on the extracellular part of $GABA_A$ receptors, because intracellularly applied steroids had no discernible effects on $GABA_A$ receptors (Lambert et al., 1990). In addition, the stringent structural requirements and the nanomolar potencies of steroids in the presence of GABA argue in favor of a specific action at the receptor proteins.

F. The Interaction of Avermectin B_1a with γ-Aminobutyric $Acid_A$ Receptors

AVM (fig. 3) is a macrocyclic lactone from *Streptomyces avermitilis* with potent insecticidal and anthelmintic actions (Olsen, 1982; Payne and Soderlund, 1993). Electrophysiological studies demonstrated that AVM and its structural analogues exert complex effects on chloride permeability in vertebrate and invertebrate nerve and muscle membranes. Some, but not all, of these effects seem to be mediated by the $GABA_A$ receptor chloride ionophor complex, at which AVM acts as an apparent partial agonist, antagonist, or allosteric modifier of the action of GABA, depending on the species, preparation and assay methodology used (Payne and Soderlund, 1993).

AVM, because of its highly hydrophobic properties, is a difficult compound to work with, because it binds to glass or plastic tubes and is thus lost from the incubation solution during the assay or during its storage in aqueous buffer solutions (Drexler and Sieghart, 1984b). This is the reason why in vitro dose-response curves obtained by different authors cannot be easily compared. Nevertheless, using special precautions (Drexler and Sieghart, 1984b), some studies indicated that there is a high affinity binding site for [^{3}H]AVM on brain membranes (Pong and Wang, 1980; Drexler and Sieghart, 1984b) and that this binding site exhibits a series of complex allosteric interactions with binding sites for GABA, benzodiazepines, barbiturates or TBPS (Olsen, 1982; Drexler and Sieghart, 1984a, b, c). Thus, depending on the concentration and conditions used, AVM stimulated or inhibited high affinity [^{3}H]GABA or [^{3}H]flunitrazepam binding (Supavilai and Karobath, 1981a; Pong and Wang, 1982; Olsen and Snowman, 1985). AVM was able to stimulate [^{35}S]TBPS binding, and high affinity [^{3}H]AVM binding was modulated by $GABA_A$ receptor agonists and antagonists in a chloride ion-dependent way (Drexler and Sieghart, 1984a, b, c). These results indicated a close association of AVM binding sites with the $GABA_A$ receptor complex and suggested that these AVM sites are not identical with the GABA, the benzodiazepine, the barbiturate, or the picrotoxinin-TBPS binding sites of this receptor (Drexler and Sieghart, 1984a, b, c). The relationship of the AVM binding site to the steroid or Ro5–4864 binding site (see next paragraph) has not been investigated.

G. The Interaction of Ro5–4864 with γ-Aminobutyric $Acid_A$ Receptors

Ro5–4864, the 4′-chloro-derivative of diazepam (fig. 1), at nanomolar concentrations, is a prototypical ligand for the "peripheral" benzodiazepine binding site (Verma and Snyder, 1989). At micromolar levels, however, this compound interacts with the $GABA_A$ receptor. Ro5–4864 is a potent convulsant (Weissman et al., 1983), and its convulsant effect is antagonized by barbiturates, diazepam, and other clinically useful benzodiazepines (Rastogi and Ticku, 1985).

In electrophysiological experiments, Ro5–4864 inhibited GABA-stimulated chloride flux and enhanced neuronal firing induced by TBPS (Dai and Woolley, 1991). In binding studies, it did not interact with the GABA or benzodiazepine binding site of $GABA_A$ receptors, but it reduced the binding of [^{35}S]TBPS in the absence and enhanced this binding in the presence of GABA. This effect of GABA could be blocked by bicuculline, suggest-

ing that the site of action of Ro5–4864 is linked to a GABA$_A$ receptor (Gee, 1987; Gee et al., 1988). The potency of Ro5–4864 for inhibition (in the absence of GABA) and for stimulation (in the presence of GABA) of [^{35}S]TBPS binding was similar to its potency for antagonizing the electrophysiological effects of GABA, indicating that all these actions were mediated by the same site (Gee, 1987). Collectively, the evidence points to a unique and relatively low affinity (K_D 250 nM) Ro5–4864 site that is linked to a GABA$_A$ receptor (Gee, 1987).

In addition to Ro5–4864, other compounds, such as the phenylquinolines PK 8165 and PK 9084 and the isoquinoline carboxamide derivative PK 11195, seem to modulate GABA$_A$ receptors by binding to the Ro5–4864 site (Gee, 1987). The compound PK 11195, at subnanomolar concentrations, specifically binds to the peripheral benzodiazepine binding site (Verma and Snyder, 1989) and at micromolar concentrations blocks the effects of Ro5–4864 on [^{35}S]TBPS binding. It also potentiated the electrophysiological effects of the GABA$_A$ agonist muscimol, indicating that this compound exhibits actions opposite to those of Ro5–4864 (Gee, 1987). Further studies, however, have to be performed to verify these effects and to more thoroughly investigate the possible interaction of Ro5–4864, PK 8165, PK 9084, and PK 11195 with the same site at GABA$_A$ receptors.

H. The Interaction of Zn^{2+} with γ-Aminobutyric Acid$_A$ Receptors

Zn^{2+} is an important constituent of the dietary intake of animals and humans. A considerable amount of research has demonstrated that Zn^{2+} can be found in many different tissues and is involved with multiple aspects of cellular biochemistry and membrane structure (Smart et al., 1994). Recently, evidence has accumulated that Zn^{2+} is able to modulate inhibitory and excitatory amino acid receptor ion channels. Thus, Zn^{2+} is concentrated in synaptic terminals and released by electrical activity in sufficient quantities to play a potential role in neurotransmission (Xie and Smart, 1991). Moreover, Zn^{2+} and, to a lesser extent, certain other metal cations, such as Cd^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+}, inhibited the GABA response of neurons in a variety of organisms, whereas Ca^{2+}, Mg^{2+}, or Ba^{2+} were consistently without effect when applied extracellularly (Celentano et al., 1991). In addition, inhibition of the GABA response by Zn^{2+} was partially relieved by Cd^{2+}, Ni^{2+}, Mn^{2+}, and Ba^{2+}; therefore, we would argue that these cations have lower intrinsic efficacies as allosteric inhibitors at GABA$_A$ receptors. Ba^{2+} obviously binds to the divalent cation site but lacks efficacy as an inhibitor of the GABA response (Celentano et al., 1991).

However, other data suggest that modulation of GABA$_A$ responses by Zn^{2+} critically depends not only on the type of preparation used, but also on the stage of neuronal development. Embryonic and young postnatal neurons seem to be more sensitive to Zn^{2+} inhibition of GABA responses than neurons from adult animals (Smart and Constanti, 1990: Smart, 1992). Zn^{2+} did not affect the main single-channel conductance and mean open and shut times but reduced the opening frequency of GABA-induced Cl^- channels (Smart, 1992).

Radioligand binding studies (Mackerer and Kochman, 1978; Mizuno et al., 1983) suggested the presence of a Zn^{2+} binding site at some, but not all, GABA$_A$ receptor subtypes. This site seems to be localized extracellularly and to be distinct from the GABA, the benzodiazepine, barbiturate, picrotoxin, and steroid recognition sites (Celentano et al., 1991; Smart, 1992). In addition, a variety of mono- and divalent cation effects have been reported on [^{3}H]diazepam or [^{3}H]flunitrazepam binding and its inactivation by heat (Squires and Saederup, 1982; Squires, 1986). The relationship of these effects to the Zn^{2+} binding site of GABA$_A$ receptors, however, is unclear.

I. The Interaction of La^{3+} with γ-Aminobutyric Acid$_A$ Receptors

Electrophysiological studies have indicated that La^{3+} and lanthanides stimulated GABA-mediated Cl^- currents in dorsal root ganglion cells (Ma and Narahashi, 1993b), with the efficacy increasing with the atomic number. In addition, lanthanides, at high concentrations (1 mM), were able to directly open GABA$_A$ receptor associated chloride channels (Ma and Narahashi, 1993b). Other evidence indicated that La^{3+} did not interfere with the benzodiazepine, barbiturate, or picrotoxin binding sites of GABA$_A$ receptors and seemed not to interfere with the effects of Zn^{2+} and Cu^{2+} on these neurons (Ma and Narahashi, 1993a). Furthermore, in a recent report (Im and Pregenzer, 1993), it was demonstrated that lanthanides at micromolar concentrations stimulated [^{35}S]TBPS binding to rat synaptosomal membranes in the absence of GABA with no appreciable effect on TBPS binding in the presence of GABA. This trivalent cation effect seemed to reflect a specific and direct interaction with GABA$_A$ receptors and could not be mimicked or inhibited by divalent metal ions including Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, and Cd^{2+}. On the other hand, Zn^{2+} ions inhibited TBPS binding, but its presence could not prevent stimulation of TBPS binding by La^{3+}. The effects of Zn^{2+} and La^{3+} ions on TBPS binding were additive, indicating that Zn^{2+} does not share the same binding site with La^{3+} (Im and Pregenzer, 1993).

J. The Interaction of Cl^- with γ-Aminobutyric Acid$_A$ Receptors

Because modulation of GABA$_A$ receptors influences GABA-induced chloride ion flux, a modulation by chloride of the various binding sites at the GABA$_A$ receptor is not surprising. Thus, evidence has accumulated indicating a dependence on, or a strong modulation by, Cl^-, Br^-, I^-, NO_3^-, SCN^-, or ClO_4^- of most if not all binding

sites and of allosteric interactions between binding sites of $GABA_A$ receptors (Olsen, 1982; Squires et al., 1983; Drexler and Sieghart, 1984a, b; Schumacher and McEwen, 1989). The specificity of the anion modulation of the various binding sites was proposed to represent coupling of these sites to a chloride ion channel.

Recently, it was demonstrated that organic anions, such as picrate and niflumate, potently inhibited the effects of anions on [^{35}S]TBPS or [^{3}H]benzodiazepine binding (Evoniuk and Skolnick, 1988). These findings suggest that picrate and niflumate bind with high affinity at or near an anion binding site that may not only regulate the movement of anions through GABA-gated chloride channels but also can modulate radioligand binding at this "supramolecular" complex.

K. The Interaction of Chlormethiazole, Propofol and Inhalation Anesthetics with γ-Aminobutyric Acid$_A$ Receptors

Both pharmacological and electrophysiological evidence suggest that the anxiolytic, anticonvulsant, and sedative/hypnotic chlormethiazole (fig. 2, Moody and Skolnick, 1989; Hales and Lambert, 1992) or the i.v. general anesthetic propofol (fig. 2) (Hales and Lambert, 1991; Concas et al., 1991) may exert at least some of their actions through the $GABA_A$ receptor complex. Thus, chlormethiazole (30 to 150 μM), as well as propofol (1 to 30 μM), dose-dependently potentiated GABA-activated currents by increasing the probability of the channel being in the conducting state, and this effect could not be inhibited by the benzodiazepine receptor antagonist Ro15–1788. The concentration of chlormethiazole affecting $GABA_A$ receptors was well within the blood level range (125 to 185 μM) of this compound after hypnotic doses (Moody and Skolnick, 1989). Similarly, the concentrations of propofol eliciting these effects seemed to be close to the concentrations needed for anesthesia (0.4 to 35 μM) (Franks and Lieb, 1994; Prince and Simmonds, 1992). At higher doses (3 mM chlormethiazole, 30 to 600 μM propofol), these compounds directly activated the $GABA_A$ receptor by increasing the chloride conductance of the cell membranes in a bicuculline-sensitive way. Chlormethiazole and propofol were found to allosterically inhibit the binding of [^{35}S]TBPS or to enhance the binding of [^{3}H]muscimol (Cross et al. 1989; Concas et al., 1990, 1991). Whereas chlormethiazole had no effect on pentobarbital-enhanced [^{3}H]flunitrazepam binding and inhibited [^{3}H]flunitrazepam binding at high concentrations (Moody and Skolnick, 1989), propofol in one report (Prince and Simmonds, 1992), but not in another report (Concas et al., 1991), seemed to enhance [^{3}H]flunitrazepam binding at high concentrations in a chloride-dependent way. These findings seem to indicate that these compounds can perturb the $GABA_A$ receptor complex by interacting with a site distinct from that for other sedative/hypnotics such as barbiturates, benzodiazepines, or $GABA_A$ agonists. Additional studies, however, are necessary to further clarify the mode of action of chlormethiazole and propofol and their possible interaction with the same binding site.

Recent reports indicate that clinically relevant concentrations of volatile anesthetics, such as isoflurane (EC_{50} 320 ± 20 μM) (Hall et al., 1994), halothane (fig. 2) 0.34 to 1.7 mM) (Yang et al., 1992), and enflurane (0.75 to 1.5 mM) (Yang et al., 1992), a class of general anesthetics with unique chemical structures distinct from those of the i.v. agents, also show GABA modulatory properties. In addition, it was demonstrated that volatile anesthetics are able to directly open chloride ion channels in vertebrate central neurons in culture, and this effect could be completely blocked by bicuculline and picrotoxinin (Yang et al. 1992; Longoni et al., 1993). Other studies indicated that halothane, enflurane, and isoflurane at clinically relevant concentrations (Franks and Lieb, 1994) enhance [^{3}H]flunitrazepam binding to the benzodiazepine binding site of $GABA_A$ receptors in a chloride ion-dependent way (Nakao et al., 1991; Harris et al., 1993). Similar to anesthetic barbiturates, the increase in [^{3}H]flunitrazepam binding observed with inhalational agents is effected through an increase in ligand affinity. In addition, isoflurane markedly augmented the effects of pentobarbital on [^{3}H]flunitrazepam binding through a decrease in the EC_{50}. Because the maximal effect of both agents was not different from pentobarbital alone, isoflurane and pentobarbital might have a common locus of action. However, other results seem to argue against this possibility (Harris et al., 1993).

In other studies, halothane, enflurane, and isoflurane, at clinically relevant concentrations, stereoselectively increased the high affinity binding of [^{3}H]muscimol to $GABA_A$ receptor sites (Longoni et al., 1993; Harris et al., 1994a) in mouse and rat brain membranes and enhanced the muscimol-stimulated $^{36}Cl^-$ efflux via $GABA_A$ receptors in rat brain cortical slices. In addition, halothane and enflurane increased basal $^{36}Cl^-$ efflux from rat brain slices in the absence of GABA agonists (Longoni et al., 1993).

Thus, several structurally distinct classes of anesthetics, including barbiturates (pentobarbital, secobarbital), steroids (alphaxalone, 3α-hydroxy-5α-dihydroprogesterone), etomidate, propofol, chlormethiazole, and inhalation anesthetics, although probably not interacting with the same site on $GABA_A$ receptors, share the property of potentiating $GABA_A$ receptor-gated Cl^- currents at low concentrations. At higher concentrations, all these compounds have the ability to directly open the $GABA_A$ receptor-associated Cl^- channel. Although the exact site and mechanism of action of these compounds so far is not sufficiently investigated, the overall depression of the central nervous system caused by the direct hyperpolarizing effect of these compounds probably is responsible for their anesthetic action.

L. The Interaction of Ethanol with γ-Aminobutyric Acid$_A$ Receptors

Ethanol exhibits a large variety of different actions on the nervous system. This compound not only influences membrane fluidity, neuronal electric activity, and synaptic transmission, it also seems to exhibit specific actions on voltage- and transmitter-gated ion channels (Deitrich et al., 1989). Several lines of evidence indicate that ethanol is able to potentiate GABAergic transmission. Thus, ethanol shares some properties with barbiturates and benzodiazepines, because it exhibits anticonvulsant, anxiolytic, and sedative activity; a development of cross-tolerance among these compounds has also been observed (Nakahiro et al., 1991). Ethanol potentiated GABA-mediated $^{36}Cl^-$ transport into cultured neurons (Mehta and Ticku, 1988; Nakahiro et al., 1991) and rat brain synaptoneurosomes (Suzdak et al., 1986b). In radioligand binding studies, no direct linkage between ethanol and the GABA$_A$ receptor has been established. Thus, ethanol does not alter the binding of [^{3}H]GABA or [^{3}H]benzodiazepine agonists to brain membranes. Although ethanol, at close-to-lethal concentrations, allosterically inhibits [^{35}S]TBPS binding, this effect does not correlate with the behavioural or intoxicating effects of alcohol (Ticku, 1990).

Similarly, electrophysiological data are controversial. In some systems, ethanol at low concentrations (1 to 50 mM) (Reynolds et al., 1992) potentiated GABA-induced responses; in other systems, this effect was not observed (Ticku, 1990; Nakahiro et al., 1991; Proctor et al., 1992). These and other studies indicate an interaction of ethanol with some, but not all, GABA$_A$ receptor subtypes.

M. The Interaction of Other Classes of Compounds with γ-Aminobutyric Acid$_A$ Receptors

Over the last couple of years, a variety of studies have been performed investigating a possible direct interaction of other classes of compounds with GABA$_A$ receptors. In many cases, these were single studies that so far have not been repeated by other groups and that don't provide sufficient information on the detailed mechanism of interaction of the respective compounds with GABA$_A$ receptors. Nevertheless, some of the investigated compounds might provide a lead for future investigations or for drug development, and therefore, these original reports are briefly summarized.

1. Loreclezole. Loreclezole (R 72063, (2)-[2-chloro-2-(2, 4-dichlorophenyl)-ethenyl]-1H-1,2,4-triazole) is a novel 'broad spectrum' anticonvulsant that inhibits seizure spread and increases seizure threshold in a range of animal models (Wauquier et al., 1990). In addition, this compound induced an anxiolytic-like effect in a rat conditioned emotional response test (Dawson et al., 1994). The anticonvulsant effect of loreclezole could be reversed by some benzodiazepine inverse agonists but not by the prototypical benzodiazepine binding site antagonist Ro15–1788, whereas the anxiolytic effect could neither be reversed by Ro15–1788 nor by the partial inverse agonist CGS 8216 (Dawson et al., 1994). In addition, loreclezole has negligible affinity for the benzodiazepine recognition site labeled by [^{3}H]flunitrazepam. A direct interaction of loreclezole with GABA$_A$ receptors, however, was demonstrated in recombinant receptor studies (Wingrove et al., 1994; see section III.B.4). The relationship of the loreclezole binding site to binding sites for other allosteric ligands of GABA$_A$ receptors so far is not clear.

2. Melatonin. The pineal hormone melatonin enhanced specific [^{3}H]muscimol binding to rat brain membranes by increasing the number of low affinity GABA$_A$ binding sites (Coloma and Niles, 1988). Because this effect was observed already at nanomolar concentrations of melatonin, an interaction of melatonin with GABA$_A$ receptors might have some physiological significance. Further studies, however, are necessary to determine the site of interaction and the mechanism of the modulatory effect of melatonin on GABA$_A$ receptors.

3. Polyamines. Polyamines, such as spermine, spermidine, and putrescine, at micromolar concentrations, are able to enhance the binding of [^{3}H]flunitrazepam and [^{3}H]diazepam to GABA$_A$ receptors but not that of GABA, muscimol, or Ro15–1788. After nonionic detergent (Triton X-100) treatment of membranes, however, potentiation was no longer observed, and inhibition of binding occurred at large concentrations of polyamines (Gilad et al., 1992). Further studies are necessary to investigate the mechanism of interaction of polyamines with GABA$_A$ receptors.

4. γ-Butyrolactones. Recently, it was demonstrated that γ-butyrolactones and γ-thiobutyrolactones that can either diminish or potentiate the action of GABA, depending on the position and size of their alkyl substituents, allosterically inhibit [^{35}S]TBPS binding to membranes from rat cerebral cortex (Holland et al., 1993). These compounds do not displace [^{3}H]flunitrazepam from its binding site and, in contrast to barbiturates and steroids, do not enhance benzodiazepine or muscimol binding. These and other results indicate that γ-butyrolactones and γ-thiobutyrolactones might act at a site different from the benzodiazepine, barbiturate, and steroid modulatory sites located on the GABA$_A$ receptor complex (Holland et al., 1993).

5. Antidepressants. A direct interaction of several antidepressants, such as amoxapine or mianserin, with GABA$_A$ receptors has been indicated by binding studies. Micromolar concentrations of these drugs fully or partially reversed the inhibitory action of GABA on [^{35}S]TBPS binding (Squires and Saederup, 1988), and it was speculated that convulsant side effects of these drugs might be caused by their GABA antagonism.

6. Dihydrogenated ergot compounds. Dihydrogenated ergot compounds at μM concentrations noncompetitively displaced the binding of [^{3}H]t-butylbicycloorthobenzo-

ate, a compound that binds to the picrotoxinin/TBPS binding site of $GABA_A$ receptors; GABA enhanced the displacement potency of dihydroergotoxine in a bicuculline-sensitive manner (Tvrdeic and Pericic, 1991, 1992). Because the same ergot compound prolonged pentobarbital-induced sleeping times and diminished the convulsive potency of picrotoxin in mice, it was suggested that dihydroergotoxine binds as an agonist to receptor sites involved in anticonvulsive and sedative hypnotic actions linked to $GABA_A$ receptors.

7. 1-Aryl-3-(aminoalkylidene)oxindoles. Recently, a series of 1-aryl-3-(aminoalkylidene)oxindoles was synthesized that seem to exhibit a GABAergic mode of action. These compounds had no apparent effect on GABA levels in the brain but enhanced the binding of [^{3}H]flunitrazepam in vivo (Sarges et al., 1989). Additional experiments must be performed to investigate the site of interaction of these compounds with the $GABA_A$ receptor.

8. Substituted pyrazinones. Other studies have identified substituted pyrazinones as a new class of allosteric modulators of $GABA_A$ receptors. These compounds potentiated GABA-mediated Cl^- currents in recombinant $GABA_A$ receptors. These effects were not inhibited by the benzodiazepine antagonist Ro15–1788 and were additive with those of barbiturates and neurosteroids (Im et al., 1993a).

9. Dihydroimidazoquinoxalines. A compound with a completely different mode of action was discovered when various imidazoquinoxalines were investigated as ligands for $GABA_A$ receptors (Dillon et al., 1993). These compounds were agonists or antagonists at the benzodiazepine site of recombinant $GABA_A$ receptors. A reduced form of these compounds, however, the compound U-93631 (4-dimethyl-3t-butylcarboxyl-4,5-dihydro[1, 5-a]imidazoquinoxaline), produced a unique response, in that it accelerated the decay of GABA-induced Cl^- currents without producing noticable changes in the amplitude of the currents. Results obtained so far indicate that this drug reversibly desensitizes $GABA_A$ receptors when GABA sites are occupied rather than acting as an open channel blocker. Furthermore, the binding site for this compound on $GABA_A$ receptors seems not to overlap with GABA, barbiturate, or benzodiazepine sites, because the drug effect persisted in the presence of excess ligands for these sites (Dillon et al., 1993).

10. Quinolones/Arylalkanoic acids. When it was demonstrated that the simultaneous administration of some Norfloxacin-like quinolone antibiotics, used in the oral treatment of urinary, biliary, intestinal and pulmonary tract infections, with the nonsteroidal anti-inflammatory arylalkanoic acid fenbufen led to serious convulsions in patients, the mechanism of this interaction was investigated. Quinolone antibiotics in clinical doses had no effects on the GABA-gated Cl^- currents from hippocampal pyramidal neurons but slightly suppressed the response at concentrations $> 10^{-5}$ M. Similarly, a metabolite of the arylalkanoic acid fenbufen had little effect on the GABA response at therapeutic concentrations. Coadministration of one of the quinolone antibiotics with the metabolite of fenbufen, however, suppressed the GABA- or pentobarbital-gated Cl^- current in a concentration-dependent manner (Akaike et al., 1991).

In other studies, it was demonstrated that quinolone antibiotics inhibited specific binding of [^{3}H]GABA or [^{3}H]muscimol to synaptic plasma membranes from rat brain and reversed the inhibitory effect of GABA on the binding of [^{35}S]TBPS (for review see Akaike et al., 1991). Arylalkanoic acids at high nanomolar to low micromolar concentrations selectively potentiated the GABA antagonistic effects of several pyrazinoquinolones on [^{35}S]TBPS binding. These compounds, however, did not potentiate the GABA antagonistic effects of other $GABA_A$ antagonists (Akaike et al., 1991; Yakushiji et al., 1992; Squires and Saederup, 1993). Recent experiments seem to suggest that quinolones and arylalkanoic acids interact with the $GABA_A$ receptor at nearby sites and that the binding affinity of quinolones to the $GABA_A$ receptor is largely enhanced by an intermolecular interaction with arylalkanoic acids (Akahane et al., 1994).

The reported results possibly suggest the existence of a new quinolone and arylalkanoic acid binding site on $GABA_A$ receptors. A more detailed recent study indicates that fenamates and other nonsteroidal anti-inflammatory drugs had a dual effect on GABA-activated membrane current responses. Currents elicited by low concentrations of GABA were potentiated, whereas currents elicited by high concentrations of GABA were inhibited (Woodward et al., 1994).

11. Arachidonic acid and unsaturated fatty acids. Free fatty acids are known to affect the function of many receptors and ion channels (for review see Koenig and Martin, 1992). Evidence so far accumulated suggests that unsaturated fatty acids, or arachidonic acid, cause a decrease in the muscimol and pentobarbital stimulated $^{36}Cl^-$ flux (Schwartz et al., 1988; Schwartz and Yu, 1992). Furthermore, enzymatic generation of free fatty acids or diacylglycerol with phospholipase A_2 or phospholipase C, or direct addition of phospholipids or unsaturated fatty acids to brain membrane suspensions in vitro, drastically altered the binding characteristics of various $GABA_A$ receptor ligands (Ueno and Kuriyama, 1981; Nielsen et al., 1988; Koenig and Martin, 1992; Witt and Nielsen, 1994). Thus, binding of [^{3}H]muscimol or [^{3}H]flunitrazepam was increased and binding of [^{35}S]TBPS was decreased by oleic acid or arachidonic acid. In addition, these compounds inhibited the stimulation of [^{3}H]flunitrazepam binding by GABA or pentobarbital, possibly indicating a reduced coupling between the respective binding sites in the presence of unsaturated fatty acids (Koenig and Martin, 1992). The effect of oleic acid or arachidonic acid was dose-dependent in the range of 8 to 300 μM and could not be mimicked by saturated free fatty acids. Interestingly, the oleic acid-

induced enhancement of [^{3}H]diazepam binding was completely abolished by the C-18 hydroxy fatty acids ricinelaidic acid and ricinoleic acid in membranes from cerebellum but only partially decreased in membranes from other brain regions (Witt and Nielsen, 1994), possibly reflecting a difference of GABA$_A$ receptor composition in these membranes.

It is not yet clear whether the effect of the unsaturated fatty acids are caused by a direct interaction with GABA$_A$ receptors, an indirect action at the lipid-protein boundary layer, or to a modulation of the overall membrane fluidity. In any case, endogenous arachidonic acid, which is known to be accumulated during different physiological and pathological situations, may be an important endogenous modulator of GABA$_A$ receptor function. Because arachidonic acid is an intermediate of prostaglandin synthesis, and because many nonsteroidal anti-inflammatory agents reduce prostaglandin synthesis by inhibiting the cyclooxygenase pathway, future studies must evaluate whether at least some of the effects of nonsteroidal anti-inflammatory agents (see section II.M.10 of this article) might possibly be produced by an excess of arachidonic acid.

N. Comments on the Pharmacology of γ-Aminobutyric Acid$_A$ Receptors

One of the most bewildering findings on the pharmacology of GABA$_A$ receptors is the apparent existence of a variety of different allosteric modulatory sites on these receptors, all of which exhibit complex allosteric interactions with each other. However, so far, only the GABA-, the benzodiazepine-, and the TBPS-binding sites can easily and directly be investigated by binding studies. All available evidence supports the conclusion that these three sites are distinct from each other but localized on the same supramolecular complex.

[^{3}H]AVM binding is difficult to investigate (see section II.F. of this article), and the relationship of the high affinity [^{3}H]steroid binding sites to GABA$_A$ receptors so far has not been clarified (see section II.E. of this article). All the other compounds interacting with GABA$_A$ receptors are either not available in a radiolabeled form or their potency for modulating GABA$_A$ receptors is too low to allow direct binding studies. Thus, the site of action of these compounds so far could only be investigated by studying their interaction with either the GABA-, the benzodiazepine-, or the TBPS-binding site of GABA$_A$ receptors.

In order to unambiguously decide whether a compound interacts directly or allosterically with one of these sites, kinetic and equilibrium binding studies must be performed at different concentrations of the radioactive ligand. However, the results obtained in many cases are difficult or sometimes even impossible to interpret. Thus, any deviation from a competitive inhibition of ligand binding to one of these sites could indicate an allosteric interaction but could also be caused by GABA$_A$ receptor heterogeneity (see section II.O.). A competitive inhibition could indicate a direct binding of the inhibitor at the site investigated, but it should be stressed that interactions at allosteric sites could also give rise to competitive displacement curves. Without a detailed kinetic analysis, a competitive displacement in binding therefore does not provide for the conclusion that both ligands bind to the same recognition sites. Only in a few cases have detailed kinetic analyses been performed for GABA$_A$ receptor ligands and, thus, the site of interaction with GABA$_A$ receptors of most of these compounds has not been unambiguously established.

An enhancement of binding of a radioactive ligand by the compound to be investigated in any case identifies an allosteric interaction with the respective site. As stated in section II.C. of this article, most of the studies performed so far on the interaction of compounds with TBPS binding have used nonequilibrium conditions (Maksay and Simonyi, 1986). The association rate of TBPS binding is rather slow, and compounds enhancing the action of GABA on GABA$_A$ receptors accelerate the approach to equilibrium (Maksay and Simonyi, 1986). One possible explanation for this observation is that the picrotoxinin/TBPS binding site might be localized within the chloride channel (Inoue and Akaike, 1988; Inomata et al., 1988) or else might not be easily accessible in the absence of GABA agonists (see section II.C.). The addition of compounds, changing the conformation of the GABA$_A$ receptor to the open state, then would facilitate access of [^{35}S]TBPS to its binding site and enhance its association rate. Transient enhancement of TBPS binding by these agents can thus be found if the time of incubation was insufficient to reach binding equilibrium. These "low-dose hooks" are thus artifacts caused by the application of nonequilibrium conditions of binding (Maksay and Simonyi, 1986), but they nevertheless reflect allosteric interactions at GABA$_A$ receptors.

In agreement with the notion that the high affinity TBPS binding might be associated with the 'closed' conformation of the chloride ion channel (Gee, 1988), other data have indicated that not only the association rate but also the dissociation rate of TBPS binding is accelerated by GABA and by compounds enhancing the actions of GABA on GABA$_A$ receptors (Maksay and Simonyi, 1986, 1988). With increasing concentration of these compounds, the dissociation—but not the association—rate is progressively accelerated, thus leading to an overall dissociation of TBPS from its binding sites. This observation from binding studies correlates with electrophysiological results, indicating that the onset of TBPS-block of GABA-induced chloride currents, as well as the recovery from the TBPS-block, is stimulated by GABA and that increasing GABA concentrations decrease the inhibition of Cl^- currents by TBPS (Van Renterghem et al., 1987; Yoon et al., 1993). These GABA concentration-dependent effects of TBPS in electro-

physiological experiments could indicate that GABA produces its effects by interacting with multiple GABA binding sites at the $GABA_A$ receptor. Similarly, the concentration-dependent effects of allosteric $GABA_A$ receptor agonists on TBPS binding could indicate an interaction of these compounds with multiple binding sites on $GABA_A$ receptors.

This conclusion is supported by a variety of evidence. Quench flow measurements of transmembrane $^{36}Cl^-$ flux (Cash and Subbarao, 1987a, b) and electrophysiological studies indicated that two GABA molecules interact with $GABA_A$ receptors for opening the associated chloride channel (Bormann, 1988; Macdonald and Twyman, 1992). In addition, binding studies have indicated that several separate, but partially interconvertible, GABA binding sites with different affinities (high-, low-, and very low affinity sites, see section II.A. of this article) seem to be present on the same $GABA_A$ receptor. It is quite possible that TBPS blocks GABA-induced chloride currents under conditions where not all of these GABA binding sites are occupied and that the binding of an additional GABA molecule induces a conformational change in the receptor, causing a reduction in its affinity for TBPS (Yoon et al., 1993).

Other electrophysiological studies have indicated that several $GABA_A$ receptor ligands, such as barbiturates, steroids, etazolate and etomidate, and the inhalation anesthetics, exhibit multiple effects at these receptors. At low concentrations, these compounds enhance the effects of GABA, whereas at higher concentrations, these compounds can directly open chloride ion channels in the absence of GABA, and this effect can be blocked by bicuculline (see sections II.D., II.E. and II.K. of this article). In addition, recent evidence suggests that the picrotoxinin block of GABA-induced chloride flux is mediated by two different mechanisms (Yoon et al., 1993). This points to the existence of at least two sites of interaction of these compounds with $GABA_A$ receptors. These sites could be homologous or heterologous. And finally, photolabeling studies have indicated the existence of several benzodiazepine binding sites in a single $GABA_A$ receptor complex (see section II.B. of this article).

The existence of several GABA, benzodiazepine, barbiturate, steroid, anesthetic, and convulsant binding sites within a single $GABA_A$ receptor introduces additional problems in interpreting binding studies and in determining the site of action of compounds modulating the $GABA_A$ receptor. It has to be assumed that there are not only allosteric interactions between the GABA-, the benzodiazepine-, and the TBPS-binding sites of $GABA_A$ receptors but also between the respective multiple binding sites of these ligands. Thus, for instance, the acceleration of the TBPS dissociation by barbiturates, etazolate, steroids, propofol, or chlormethiazole could indicate that these compounds bind to sites allosterically coupled to the convulsant TBPS sites. Alternatively, these compounds could compete for individual attachment points at the same binding site (Prinz and Striessnig, 1993). And finally, the possibility cannot be excluded that these compounds, by binding to one convulsant TBPS site, elicit a conformational change in other TBPS site(s) present in the same $GABA_A$ receptor, thus causing an accelerated dissociation of TBPS (Maksay and Ticku, 1985a, b).

Future experiments must decide between these possibilities. Thus, specific high affinity ligands for the postulated barbiturate-, steroid-, propofol-, or chlormethiazole-binding sites must be developed to directly investigate these sites. In addition, a thorough reinvestigation by kinetic and equilibrium binding studies of the interaction of these compounds with TBPS binding sites must be performed in recombinant $GABA_A$ receptors. The use of recombinant receptors at least will reduce the interpretation problems caused by heterogeneity of receptors in brain membranes (however, see section III.B.7.). Finally, mutagenesis studies and structural investigations of recombinant receptors might shed some light on the possible existence of separate and distinct allosteric binding sites for these compounds.

O. Pharmacological Heterogeneity of γ-Aminobutyric Acid$_A$ Receptors in Brain Tissue

So far, no direct $GABA_A$ receptor agonist or antagonist is known that is able to distinguish between possible different $GABA_A$ receptors. In addition, not many compounds interacting with the presumptive barbiturate, steroid, or anesthetic sites have been investigated in sufficient detail, and thus, no compound has been identified that possesses a significant ability to distinguish between possible different $GABA_A$ receptors. There are, however, reports on a possible heterogeneity of the GABA- (Olsen, 1982; Quast and Brenner, 1983), barbiturate/etazolate- (Olsen, 1982; Leeb-Lundberg and Olsen, 1983), steroid- (Morrow et al., 1990; Gee and Lan, 1991; Sapp et al., 1992; Prince and Simmonds, 1993), and inhalational anesthetic- (Harris et al., 1993) binding sites of $GABA_A$ receptors. These reports draw their conclusions from differences in the potency of the respective compounds for allosteric interaction with $GABA_A$ receptors in different brain regions.

In contrast to most of the other allosteric sites on $GABA_A$ receptors, the benzodiazepine binding site can be easily investigated by binding studies and has attracted a lot of interest because of its clinical significance. This is the reason most of the additional evidence for a pharmacological heterogeneity of $GABA_A$ receptors involves the benzodiazepine binding site of these receptors.

Because the classical benzodiazepines had a similar affinity for benzodiazepine receptors in all brain regions investigated (Braestrup and Nielsen, 1983), it was assumed originally that there is no heterogeneity of $GABA_A$-benzodiazepine receptors. However, in the last

couple of years, several compounds with distinct chemical structures have been identified that seemed to differentially interact with benzodiazepine binding sites of GABA$_A$ receptors in various brain regions (fig. 4). Thus, it has been demonstrated that the triazolopyridazine Cl 218872 (Klepner et al., 1979), some β-carbolines such as βCCM, βCCE or βCCP esters of β-carboline-3-carboxylate (Nielsen and Braestrup, 1980), some benzodiazepines such as quazepam or cinolazepam and their metabolites (Sieghart, 1983; Sieghart and Schuster, 1984), and the imidazopyridines zolpidem or alpidem (Arbilla and Langer, 1986) exhibit affinities for benzodiazepine receptors in cerebellum several times higher than for those in hippocampus and other brain regions (Sieghart, 1989). These and other results indicating a coupling of these benzodiazepine binding sites with GABA$_A$ receptors (Regan et al., 1981; Arbilla et al., 1986; Corda et al., 1988) supported the existence of at least two GABA$_A$ receptors associated with benzodiazepine binding sites: a GABA$_A$-BZ$_1$ receptor enriched in cerebellum and exhibiting a high affinity for the compounds mentioned above, and a GABA$_A$-BZ$_2$ receptor enriched in hippocampus and some other brain regions and exhibiting a low affinity for these compounds (Sieghart, 1989).

The differential regional distribution of the BZ$_1$ and BZ$_2$ binding sites was confirmed and further investigated by a variety of autoradiographic (Scott Young et al., 1981; Unnerstall et al., 1982; Niddam et al., 1987; Dennis et al., 1988) and lesion studies (Lo et al., 1983; Corda et al., 1986a). More recently, radioligand binding and autoradiographic studies pointed to a more extensive heterogeneity of GABA$_A$ receptor-associated central benzodiazepine binding sites (Olsen et al., 1990; Massotti et al., 1991; Sieghart and Schlerka, 1991; Maguire et al., 1992).

In other studies, a differential distribution of GABA- and benzodiazepine- (Placheta and Karobath, 1979; Biggio et al., 1980; Unnerstall et al., 1981), and benzodiazepine- and TBPS-binding sites (Gee et al. 1983; Wamsley et al., 1983) has been observed that was especially obvious in cerebellum. These data indicated that GABA$_A$ receptors do exist that either do not have benzodiazepine or TBPS-binding sites or exhibit a low affinity for these compounds and thus cannot be identified by autoradiography. Recent evidence indicates that these receptors seem to be especially abundant in neonatal neurons (Rovira and Ben-Ari, 1991).

Additional heterogeneity of GABA$_A$ receptors is inferred from the fact that Zn^{2+} (Smart and Constanti, 1990; Smart, 1992) and ethanol (Ticku, 1990; Nakahiro et al., 1991; Proctor et al., 1992) were able to inhibit or enhance the GABA-induced chloride ion flux of some, but not all, GABA$_A$ receptors in the brain, respectively. Thus, the various allosteric binding sites are not necessarily present on all GABA$_A$ receptors.

III. Molecular Biology of γ-Aminobutyric Acid$_A$ Receptors

A. Molecular Structure of γ-Aminobutyric Acid$_A$ Receptor Subunits

The existence of distinct GABA$_A$-benzodiazepine receptors is supported by molecular biological studies. GABA$_A$ receptors were purified from brain membranes by affinity chromatography (Sigel and Barnard, 1984), and the purified proteins were partially sequenced. Screening of brain cDNA libraries with oligonucleotide probes constructed according to the sequence information obtained led to the identification of a variety of structurally related GABA$_A$ receptor subunits (Schofield et al., 1987; Olsen and Tobin, 1990; Burt and Kamatchi, 1991). So far, a total of 6α-, 3β-, 3γ-, 1δ-, and 2ρ-subunits of the GABA$_A$ receptor have been cloned and sequenced from mammalian brain. In addition, alternatively spliced forms (γ_{2S} and γ_{2L}) of the γ_2-subunit (Whiting et al., 1990; Kofuji et al., 1991) and the α_6 subunit (Korpi et al., 1994) have been identified. Furthermore, a fourth γ-subunit (Harvey et al., 1993) and two alternatively spliced forms of a fourth β-subunit (Bateson et al., 1991) have so far been identified in the brains of chickens only.

Each of these protein subunits consists of a large hydrophilic NH_2-terminal part with several potential glycosylation sites and a cystine loop formed by two conserved cysteines. This part is followed by four putative transmembrane domains and a large intracellular loop between the third and fourth transmembrane domain, which contains possible phosphorylation sites (Olsen and Tobin, 1990; Burt and Kamatchi, 1991). The amino acid homology of the various subunit classes is approximately 30 to 40%. Within a subunit class, the various members exhibit homologies in their amino acid sequences of about 60 to 80%.

Each subunit is encoded by a separate gene, and the various genes encoding for the individual subunits cluster on different chromosomes. Thus, the genes encoding for the human α_2-, β_1-, and γ_1-subunits have been localized on chromosome 4, and those for the α_1-, α_6-, β_2- and γ_2-subunit are colocalized on chromosome 5 (Schantz-Wilcox et al., 1992; Hicks et al., 1994). The genes for the α_5- and β_3-subunits have been localized on the human chromosome 15 and are separated from each other by less than 100 kb (Sinnett et al., 1993). In the mouse, the genes encoding for the α_5- and β_3-subunits, together with the genes for the α_4- (Danciger et al., 1993) and γ_3-subunit (Nakatsu et al., 1993), have been localized on chromosome 7, which corresponds with the human chromosome 15. The genes for ρ_1- and ρ_2-subunits are localized on human chromosome 6 (Cutting et al., 1992), and the genes for the α_3- (Derry and Barnard, 1991) or δ-subunit (Sommer et al., 1990) have been localized on chromosome X or chromosome 1, respectively.

Because of the homology in their amino acid sequence and in the structure of their subunits, it was concluded

that the $GABA_A$ receptors (Schofield et al., 1987), the nicotinic acetylcholine receptors (Boulter et al., 1987), some glutamate receptors (Kutsuwada et al., 1992), and the strychnine-sensitive glycine receptors (Grenningloh et al., 1987) are members of a superfamily of ligand-gated ion channels. In analogy to the nicotinic acetylcholine receptor, it is therefore assumed that five subunits are necessary for the formation of GABA-induced Cl^- channels, and electron-microscopic image analysis performed on purified $GABA_A$ receptors seems to support this conclusion (Nayeem et al., 1994).

B. Pharmacology of Recombinant γ-Aminobutyric Acid$_A$ Receptors

1. Model systems for the investigation of γ-aminobutyric acid$_A$ receptors. Several expression systems have been used for the investigation of recombinant $GABA_A$ receptors. The direct injection into *Xenopus* oocytes of mRNA encoding for $GABA_A$ receptor subunits is a relatively rapid and simple way to elicit the biosynthesis of these receptors that then can be investigated by electrophysiological techniques (Levitan et al., 1988b; Sigel et al., 1990). This technique has the advantage that each oocyte investigated, after injection, contains the genetic information for all the desired subunits. Nevertheless, some variability in the responses of the oocytes has been observed, indicating that the expression of $GABA_A$ receptor subunits vary in different oocytes.

In contrast to *Xenopus* oocytes that have to be singly injected and from which not enough membranes for receptor binding studies can be obtained, mammalian or insect cells transfected with the respective $GABA_A$ receptor subunits can be investigated by binding studies as well as by electrophysiological techniques. So far, most of the studies have been performed with HEK cells (HEK 293 cells, American Type Culture Collection CRL 1573) (Verdoorn et al., 1990; Knoflach et al., 1992). However, other cells, such as mouse fibroblasts (Horne et al., 1993), chinese hamster ovary cells (Porter et al., 1992) or insect cells (Atkinson et al., 1992; Pregenzer et al., 1993), have also been used for recombinant $GABA_A$ receptor studies.

After transient transfection, only 20 to 60% of the transfected cells actually express $GABA_A$ receptors (Knoflach et al., 1992; Verdoorn et al., 1990: Moss et al., 1991), and it is possible that some of the cells do not contain all the subunits used for transfection. Thus, the composition of recombinant receptors in transfected cells possibly is more heterogenous than in *Xenopus* oocytes. In addition, working with transiently transfected mammalian cells is quite cumbersome because of the slow growth of cells, the variable expression of receptors, and the long incubation period required between transfection and harvesting of the cells.

Therefore, stably transfected cell lines containing $\alpha_1\beta_1$ (Porter et al., 1992), $\alpha_1\beta_2$ (Valeyev et al., 1993), $\alpha_1\beta_3$ (Valeyev et al., 1993), $\alpha_1\gamma_2$ (Wong et al., 1992), $\alpha_1\beta_1\gamma_{2L}$ (Hadingham et al., 1992), $\alpha_1\beta_2\gamma_2$ (Carter et al., 1992; Im et al., 1992; Hamilton et al., 1993), or $\alpha_3\beta_2\gamma_2$ (Carter et al., 1992) subunits have recently been produced. However, the expression efficiency of recombinant receptors in these cell lines in most cases was low (Hadingham et al., 1992; Wong et al., 1992; Hamilton et al., 1993). Only in insect cells were B_{max} values of [^{3}H]flunitrazepam binding slightly higher than those found in the brain (Carter et al., 1992). However, even in stably transfected cells, $GABA_A$ receptors were not found on every cell investigated (Valeyev et al., 1993). This might have been caused by a slow loss of $GABA_A$ receptor subunits or by an ineffective or changed receptor assembly in part of the cells.

An alternative to stably transfected cells are cell lines endogenously expressing a defined set of $GABA_A$ receptor subunits. Recently, several such cell lines have been identified. Thus, the neuronal-like cell lines B 35, B 65, B 103, and B 104 seem to contain the α_1-subunit mRNA as demonstrated by polymerase chain reaction and by Northern and Western blots. However, [^{3}H]muscimol binding could be measured only in the B 65 cells, and none of these cells exhibited GABA-stimulated chloride conductance as measured by the patch clamp technique (Kasckow et al., 1992). These results were confirmed in a recent paper demonstrating the presence of 13 $GABA_A$ receptor mRNAs in a total of 13 cell lines derived from diverse tissue origins (Tyndale et al., 1994). All cell lines examined contained detectable levels of at least one—but in many cases of several—$GABA_A$ receptor subunit mRNAs. Only two of these cell lines (the RINm5F or βTC3 cell line from the endocrine pancreas), however, exhibited GABA-evoked currents in the whole cell configuration of the patch clamp technique (Hales and Tyndale, 1994). Many factors might contribute to why cell lines with subunit mRNAs are not producing detectable channels. These include insufficient amounts of mRNAs, defective mRNAs, lack of subunit translation, incorrect subunit combinations, or inability to assemble or localize channels correctly. Alternatively, functional $GABA_A$ receptors are expressed in these cells below the level of detection.

In another report, the presence of mRNAs encoding α_1-, β_1-, and β_3-subunits of $GABA_A$ receptors in immortalized hypothalamic GT 1–7 neurons has been demonstrated (Hales et al., 1992). In addition, these cells exhibit GABA-activated chloride currents. Similarly, a functional $GABA_A$ receptor which is not modulated by benzodiazepines has been identified in the human neuroblastoma IMR-32 cell line. Preliminary characterization identified the presence of the α_3-subunit of $GABA_A$ receptors in this cell line (Noble et al., 1993). The possible presence of additional $GABA_A$ receptor subunits in the IMR-32 cells must be further investigated.

In a recent report, it was demonstrated that neuronal cells derived from the EC cell line P19 express mRNAs for various α- and β-subunits as well as for the γ_2-

subunit of the GABA$_A$ receptor chloride channel complex. Whole-cell voltage clamp recording revealed that these cells possess GABA receptor-activated chloride currents that are blocked by bicuculline and potentiated by the benzodiazepine flurazepam (Reynolds et al., 1994). P19 EC cells thus represent a stable neuronal cell line that expresses functional receptors with all the characteristics of native GABA$_A$ receptors. However, because of the presence of several α- and β-subunits in these cells, the GABA$_A$ receptors expressed by these cells probably are heterogeneous.

2. Properties of receptors consisting of a single subunit. Electrophysiological studies have indicated that *Xenopus* oocytes, embryonic kidney cells, or insect cells expressing only single GABA$_A$ receptor subunits are able to form homo-oligomeric chloride ion channels, at least some of which can be activated by rather high concentrations of GABA and can be inhibited by bicuculline (Blair et al., 1988; Pritchett et al., 1988; Shivers et al., 1989; Verdoorn et al., 1990; Joyce et al., 1993). Currents activated by 10 μM GABA, however, were an order of magnitude smaller than those activated in cells transfected with most dual or triple subunit combinations (Verdoorn et al., 1990), indicating either an infrequent channel opening or a low efficiency of expression and assembly of single subunits. This might have been one reason why sometimes no GABA-activated chloride ion channels could be detected after transient transfection of cells with single GABA$_A$ receptor subunits (Sigel et al., 1990). Species differences might have been another reason. Thus, it has been demonstrated that homo-oligomeric rat β_1-subunit-containing channels could be formed in *Xenopus* oocytes, but the channels seemed to be open in the absence of GABA (Sigel et al., 1989). In contrast, human β_1-subunits expressed in HEK 293 cells (Pritchett et al., 1988), or bovine β_1-subunits expressed in SF9 insect cells (Joyce et al., 1993), produced GABA-gated channels that could be blocked by bicuculline.

Homo-oligomeric channels consisting of either α-, β-, γ_2-, or δ- subunits exhibited multiple conductance states and showed desensitization (Shivers et al., 1989; Verdoorn et al., 1990; Sigel et al., 1990; Burt and Kamatchi, 1991). In addition, GABA-induced chloride flux could be inhibited by picrotoxinin and stimulated by barbiturates and possibly steroids (Shivers et al., 1989; Puia et al., 1990; Atkinson et al., 1992). These observations indicate that GABA-, picrotoxinin-, barbiturate- and possibly steroid-binding sites either are constitutively present on each of these subunits, or can form on assembly of homo- or hetero-oligomeric channels (table 1). Thus, depending on steric requirements, up to five binding sites for GABA, barbiturates, and steroids might be present on GABA$_A$ receptors, and this might be the molecular basis for the observed multiplicity of these sites in a single GABA$_A$ receptor (see sections II.A. and II.N. of this article).

These electrophysiological data are supported by the recent observation that homo-oligomeric GABA$_A$ receptors consisting of rat β_3-subunits exhibited high affinity [^{35}S]TBPS-binding that could be modulated by pentobarbital, etazolate, etomidate, alphaxalone, propofol, chlormethiazole, and Ro5–4864 (Slany et al., submitted

TABLE 1
Binding sites present on recombinant GABA$_A$ receptors with different subunit compositions

	Subunit composition of recombinant receptors								
	α	β	γ	δ	ρ	αβ	βγ	αγ	αβγ
Electrophysiological effects									
GABA	+	+	+	+	+	+	+	+	+
Bicuculline	+	+	+	+	−	+	+	+	+
Picrotoxin	+	+	+	+	+	+	+	+	+
Pentobarbital	+	+	+	+	−	+	+	+	+
Alphaxalone	nd	+	nd	nd	nd	+	+	nd	+
Propofol	nd	+	nd	nd	nd	+	nd	nd	+
Chlormethiazole	nd	nd	nd	nd	nd	+	nd	nd	+
Inhalation anesthetics	nd	nd	nd	nd	−	+	nd	+	+
Zn^{2+}	+	+	−	nd	nd	+	−	−	−
La^{3+} (high affinity)	nd	nd	nd	nd	nd	−	nd	nd	+
Ro5–4864	−	−	−	−	nd	−	+	+	+
Benzodiazepines	−	−	−	−	−	−	+	+	+
Activity in binding studies									
[^{3}H]muscimol	−	−	−	nd	nd	+	−	−	+
[^{35}S]TBPS	−	+	−	nd	nd	+	+	−	+
[^{3}H]Benzodiazepines	−	−	−	nd	nd	−	−	+	+
Pentobarbital	np	+	np	nd	nd	+	+	+	+
Alphaxalone	np	+	np	nd	nd	+	+	+	+
Propofol	np	+	np	nd	nd	+	+	+	+
Chlormethiazole	np	+	np	nd	nd	+	+	+	+
Ro5–4864	np	+	np	nd	nd	+	+	nd	+

nd, not determined; np, not possible.

for publication). Thus, binding sites for all these compounds seem to be present on homo-oligomeric receptors formed from β_3-subunits (table 1). These data are supported by a recent report indicating that cDNA encoding for the human β_1-subunit injected into *Xenopus* oocytes gave rise to GABA-activated chloride ion channels that could be directly opened by pentobarbital or propofol in the absence of GABA (Sanna et al., 1994). Interestingly, however, no specific [^{3}H]muscimol binding could be observed in homo-oligomeric receptors consisting of β_3-subunits, and [^{35}S]TBPS binding could not be modulated by GABA in these receptors (Slany et al., submitted for publication). The absence of a GABA or [^{3}H]muscimol binding site on homo-oligomeric β_3-receptors might be related to the observation that rat β_1-subunit containing channels seemed to be open in the absence of GABA (Sigel et al., 1989) and could be caused by a species difference as discussed above (Pritchett et al., 1988; Joyce et al., 1993). The presence of high affinity [^{35}S]TBPS binding sites on homo-oligomeric $GABA_A$ receptors consisting of β_3-subunits, but not on those consisting of α_1- or γ_2-subunits (Slany et al., submitted for publication), seems to indicate, that picrotoxinin and [^{35}S]TBPS, although possibly binding to the same site, have different structural requirements.

The homo-oligomeric channels could not, however, be modulated by benzodiazepines (table 1), and, because of their inefficient assembly, it is rather unlikely that receptor subtypes consisting of a single subunit type are formed in the brain. In addition, because of the low density of receptors in cells transfected with single subunits and because of some inconsistencies in detection of single-subunit channels (Sigel et al., 1990), not many of these single-subunit receptors were investigated, and these constructs, thus, have not been adequately characterized.

3. Properties of receptors consisting of two different subunits. Channels containing two different subunits formed more efficiently and could be activated by lower GABA concentrations; the induced Cl^- ion fluxes were higher than in homo-oligomeric channels (Sigel et al., 1990; Knoflach et al., 1992). There seem, however, to be differences in the expression efficiency of dimeric subunit combinations. Thus, the highest level of expression was found for $\alpha_1\gamma_2$-, $\alpha_1\beta_2$-, and $\alpha_1\beta_2\gamma_2$-subunit combinations. Cells transfected with single subunit cDNAs or $\beta_2\gamma_2$-combinations also expressed functional receptors, but the level of expression was much lower (Draguhn et al., 1990; Verdoorn et al., 1990; Sigel et al., 1990). Similar results were obtained with various α_3-, β_1-, and γ_2-subunit combinations (Knoflach et al., 1992). These results might indicate that $GABA_A$ receptors are assembled from $\alpha\beta$ and/or $\alpha\gamma$ dimers and that free subunits are more likely to be incorporated into these preferred combinations than to assemble to form homo-oligomers. These results were only partially confirmed by other investigators (Angelotti et al., 1993a), who demonstrated that functional $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ $GABA_A$ receptors were assembled in transfected mouse L929 fibroblast cells but that $\alpha_1\gamma_{2S}$ and $\beta_1\gamma_{2S}$ $GABA_A$ receptors were not expressed. Differences in the subunit types or species differences in the subunits used for transfection might have caused this discrepancy. Alternatively, this discrepancy might have been caused by the use of different model systems (*Xenopus* oocytes, HEK 293 cells, L929 cells), with inherent differences in the assembly of $GABA_A$ receptors (Angelotti et al., 1993a). In addition, the overall subunit expression efficiency of the model system used might determine the subunit combinations which are formed in the cells (see section III.B.7. of this article). Although $\alpha\beta$-dimers might be preferentially formed as intermediates of $GABA_A$ receptor assembly at a low level of expression of subunits, at a high level of subunit expression, energetically possibly less stable $\alpha\gamma$- or $\beta\gamma$- dimers might be formed and might then assemble to $GABA_A$ receptors consisting of $\alpha\gamma$- or $\beta\gamma$-subunits.

Single-channel recordings indicated that, similar to homomeric channels, these dimeric channels exhibited multiple conductance states. The main-conductance level of $\alpha_1\beta_x$ $GABA_A$ receptor channels, however, was smaller than that of $\alpha_1\beta_x\gamma_2$ receptors (Verdoorn et al., 1990; Angelotti and Macdonald, 1993). As homomeric channels, dimeric channels showed desensitization, but GABA in most cases exhibited no cooperativity in gating the channels (Sigel et al., 1990).

The particular α-subunit present in dimeric channels affected the functional properties of recombinant receptors. Thus, receptor complexes formed with either α_1-, α_2-, or α_3- and the β_1-subunit, displayed up to a 30-fold difference in sensitivity to GABA (Levitan et al., 1988a). In addition, the $\alpha_5\beta_1$ combination seemed to be more sensitive to GABA than the $\alpha_1\beta_1$ or $\alpha_3\beta_1$ combinations (Sigel et al., 1990). There may, however, be differences between species, in that rat $\alpha_1\beta_1$ or $\alpha_3\beta_1$ combinations display similar GABA dose-response curves (Malherbe et al., 1990), whereas the equivalent bovine combinations show a three-fold difference in GABA sensitivity (Levitan et al., 1988a). Alternatively, these differences might have been caused by a point mutation in the α_1-subunit of the $GABA_A$ receptor, influencing GABA-sensitivity of recombinant receptors (Sigel et al., 1990, 1992; see section III.B.7.).

GABA-induced chloride ion flux in all dimeric channels was inhibited by picrotoxin and bicuculline and enhanced by barbiturates (Burt and Kamatchi, 1991) (table 1). There were, however, pronounced functional differences between recombinant receptors containing $\alpha_1\gamma_2$-, $\alpha_1\beta_2$-, or $\beta_2\gamma_2$-subunit combinations (Sigel et al., 1990; Verdoorn et al., 1990). In addition, the presence of allosteric modulatory sites on these receptors seems to depend on the subunit composition. Thus, the presence of α- and β-subunits seems to be necessary to produce detectable levels of [^{3}H]muscimol binding sites in $GABA_A$ receptors (Pritchett et al., 1988; Pregenzer et al.,

1993). Recombinant receptors consisting of single subunits or $\alpha_1\gamma_2$- or $\beta_2\gamma_2$-subunit combinations seem either not to contain these binding sites (table 1) or seem to have been expressed rather inefficiently in the experimental system used in this study (Pregenzer et al., 1993).

As mentioned in section III.B.2., [^{35}S]TBPS binding sites have been identified on all GABA$_A$ receptors containing β_3-subunits. Thus, homo-oligomeric receptors containing β_3-subunits, as well as receptors consisting of $\alpha_1\beta_3$-, $\beta_3\gamma_2$-, or $\alpha_1\beta_3\gamma_2$-subunits exhibited high affinity [^{35}S]TBPS binding, and the potency of pentobarbital, etazolate, etomidate, alphaxalone, propofol, chlormethiazole, and Ro5–4864 to modulate [^{35}S]TBPS binding depended on the subunit combination investigated (Zezula et al., 1994). A difference in the potency of several allosteric GABA$_A$ receptor ligands for modulation of [^{35}S]TBPS binding was also observed in recombinant $\alpha\beta$- or $\alpha\beta\gamma_2$-receptors containing different α-subunits (Im et al., 1994).

Enhancement by steroids of GABA-induced chloride ion flux, as well as direct opening of chloride channels at higher steroid concentrations, has been demonstrated in homo- and hetero-oligomeric channels containing β_1-subunits (Puia et al., 1990). The efficacy of steroids to enhance GABA-induced Cl^- ion flux in hetero-oligomeric channels depended on the subunit combination investigated (Shingai et al., 1991; Zaman et al., 1992).

The presence of α- and β-subunits seems to be sufficient to produce binding sites for propofol and chlormethiazole, because preliminary evidence indicates that propofol (Hales and Lambert, 1991) or chlormethiazole (Hales and Lambert, 1992) are able to enhance GABA-induced Cl^- flux in Chinese hamster ovary cells cotransfected with α_1- and β_1-subunits.

Similarly, isoflurane or enflurane were able to stimulate GABA-activated current in receptors containing $\alpha_1\beta_1$- or $\alpha_2\beta_1$-subunits (Lin et al., 1993; Harrison et al., 1993). The presence of the γ_2-subunit decreases the sensitivity to enflurane in that enflurane effects were significantly smaller with the $\alpha_1\beta_1\gamma_{2S}$- or $\alpha_1\beta_1\gamma_{2L}$-combinations than with the $\alpha_1\beta_1$-combination (Lin et al., 1993). In addition, it was demonstrated that although receptors consisting of α_2- and γ_2-subunits were poorly expressed, they were sensitive to isoflurane (Harrison et al., 1993).

A Zn^{2+} binding site seems to be present on channels that contain only α_1- or β_2- or a combination of these subunits. GABA-induced current in these channels was blocked by Zn^{2+}, whereas channels that contained the γ_2-subunit alone or in combination with α- or/and β-subunits were rather insensitive to Zn^{2+} (Draguhn et al., 1990). The sensitivity of some but not all GABA$_A$ receptors to Zn^{2+} in the brains of newborn and adult rats (Smart and Constanti, 1990) thus could indicate the occurrence in the brain of GABA$_A$ receptors containing only α- and β-subunits.

GABA-mediated chloride currents in recombinant receptors consisting of $\alpha_1\beta_2$-subunits were only weakly stimulated by La^{3+} ions at high concentrations (EC_{50} near 200 μM). In contrast, La^{3+} dose-dependently potentiated the GABA-induced chloride current in the $\alpha_1\beta_2\gamma_2$-subtype with an EC_{50} of 21.3 μM (Im et al., 1992). This indicates that the $\alpha_1\beta_2$-subtype is lacking a high affinity site for La^{3+}. This site might either be located directly on the γ-subunit or the presence of a γ-subunit in GABA$_A$ receptors might cause an increase in the affinity of the La^{3+} binding site on α- or β-subunits. This selectivity of La^{3+} is quite opposite to that of Zn^{2+}, which inhibited the GABA response in the $\alpha_1\beta_2$-subtype with only a marginal action on the $\alpha_1\beta_2\gamma_2$-subtype (see paragraph above). In the dorsal root ganglion neurons, GABA responses measured in the whole cell configuration of the patch clamp technique were both potentiated and inhibited by La^{3+} and Zn^{2+}, respectively (Ma and Narahashi, 1993a), and this could be interpreted to mean that $\alpha_1\beta_2$- and $\alpha_1\beta_2\gamma_2$-type receptors do exist together in these cells.

Electrophysiological studies have indicated that at least two different subunits, one of which is a γ_1- or a γ_2-subunit, seem to be necessary for the formation of Ro5–4864 binding sites (Puia et al., 1989, 1991). Thus, Ro5–4864 inhibited GABA-induced ion flux in all recombinant receptors containing γ_1- or γ_2-subunits in dual or triple combinations with α- or/and β-subunits. This compound, however, was inactive at channels composed of α_1- and β_1-subunits (Puia et al., 1989). These studies are in contrast to a more recent study indicating that Ro5–4864 was able to inhibit [^{35}S]TBPS binding in all recombinant GABA$_A$ receptors containing β_3-subunits (Zezula et al., 1994). These discrepancies might have been caused by differences in the β_1- and β_3-subunit of GABA$_A$ receptors, by the use of different expression systems, by a weak expression of the α_1- or β_1-subunit under the conditions of Puia et al. (1989), or by the possibility that Ro5–4864 is able to inhibit [^{35}S]TBPS binding but not to inhibit GABA-induced ion flux in receptors composed of α_1- and β_1-subunits.

Channels produced by expression of only one or two different GABA$_A$ receptor subunits in most cases showed no or an atypical response to benzodiazepine receptor ligands (Sigel et al., 1990; Knoflach et al., 1992). In several reports, however, it has been demonstrated that an $\alpha\gamma$-subunit composition is sufficient to produce GABA-gated chloride ion currents that are augmented by benzodiazepines and are inhibited by the inverse benzodiazepine receptor agonist DMCM (Puia et al., 1989; Knoflach et al., 1992; Wong et al., 1992). [^{3}H]benzodiazepine binding sites have been detected on recombinant receptors consisting of $\alpha_1\gamma_2$-subunits, and the binding characteristics were similar to those of receptors found in cerebellar membranes (Wong et al., 1992; Slany et al., 1994). Other results indicated that the $\beta_2\gamma_2$-receptor subtype displays GABA-induced Cl^-

currents that are potentiated by triazolam and other nonselective benzodiazepine receptor ligands, such as diazepam and zopiclone. In contrast to receptors containing $\alpha_1\gamma_2$- or $\alpha_1\beta_2\gamma_2$ subunits, in receptors containing $\beta_2\gamma_2$-subunits, the BZ_1 benzodiazepine receptor ligands, zolpidem, alpidem, and Cl 218872, showed no or very low levels of potentiation of GABA-induced Cl^- flux (Sigel et al., 1990; Im et al., 1993b). These data indicate that, in the presence of γ_2-subunits, β_2-subunits may substitute for α_1-subunits in forming the benzodiazepine binding site of limited sensitivity to the type I ligands. These results indicate that benzodiazepine binding sites are present on recombinant receptors containing $\alpha\gamma$- and $\beta\gamma$- (Puia et al., 1989; Im et al., 1993b) subunits.

All the data discussed so far are summarized in table 1. They indicate that $GABA_A$ receptors consisting of α- and β-subunits are activated by GABA and inhibited by bicuculline and picrotoxin. These receptors seem to contain [^{3}H]muscimol and [^{35}S]TBPS binding sites and seem to be modulated by barbiturates, steroids, propofol, chlormethiazole and the inhalation anesthetics and seem to be inhibited by Zn^{2+}. Receptors consisting of α- and β-subunits, however, seem not to have benzodiazepine or high affinity La^{3+} binding sites and possibly are not inhibited by Ro5–4864 (table 1).

Receptors containing α- and γ-subunits similarly are activated by GABA and inhibited by bicuculline and picrotoxin. GABA-induced chloride ion flux in these receptors is stimulated by barbiturates and the inhalation anesthetics and is inhibited by Ro5–4864, but not by Zn^{2+}. No information is available on the effect of steroids, propofol, and chlormethiazole on recombinant receptors consisting of $\alpha\gamma$ subunits (table 1).

Binding studies indicated that receptors consisting of α- and γ-subunits seem not to exhibit high affinity [^{3}H]muscimol or [^{35}S]TBPS binding sites (Pregenzer et al., 1993; Zezula et al., 1994). These sites, thus, might either be absent in these receptors or might exhibit a low affinity for [^{3}H]muscimol or [^{35}S]TBPS. Because electrophysiological studies indicated the presence of GABA and picrotoxin sites on $\alpha\gamma$ receptors, the steric requirements for GABA- and muscimol- or for TBPS- and picrotoxin binding might be different (table 1). Receptors consisting of α- and γ-subunits, however, seem to contain [^{3}H]flunitrazepam binding sites, and the GABA-induced chloride ion flux of these receptors could be modulated by benzodiazepines. The observation that [^{3}H]flunitrazepam binding to these receptors could be inhibited by pentobarbital, alphaxalone, propofol, and chlormethiazole (Slany et al., 1994) indicates that binding sites for the respective compounds seem to be present on receptors consisting of $\alpha\gamma$ subunits.

Receptors consisting of β- and γ-subunits seem to be formed rather inefficiently but seem to exhibit properties similar to those of receptors containing $\alpha\gamma$-subunits. Recombinant $\beta\gamma$ receptors, however, in contrast to recombinant $\alpha\gamma$ receptors, exhibited a high affinity [^{35}S]TBPS binding, which could be inhibited by pentobarbital, alphaxalone, propofol, chlormethiazole, and Ro5–4864 (Zezula et al., 1994; table 1). No information is available on dual receptor combinations containing δ- or ρ-subunits.

4. Properties of receptors consisting of three different subunits. A coexpression of α-, β-, and γ_2-subunits resulted in large GABA-gated Cl^- currents that could be inhibited by bicuculline, picrotoxin and Ro5–4864 and could be stimulated by La^{3+}, pentobarbital, steroids, and inhalation anesthetics (table 1) (Puia et al., 1989, 1990, 1991; Sigel et al., 1990; Verdoorn et al., 1990; Im et al., 1992; Harrison et al., 1993; Lin et al., 1993). Cooperativity of GABA for channel gating was apparent in most of these receptors, and, as with receptors containing two different subunits, the GABA responsiveness and the conductance properties, as well as the potency and efficacy of the various compounds allosterically modulating GABA-induced ion flux, varied with the type of α-, β-, or γ-subunit present in the receptors (Sigel et al., 1990; Lan et al., 1991; Shingai et al., 1991; Harrison et al., 1993; Puia et al., 1993). Furthermore, a robust modulation by benzodiazepine receptor ligands was generally observed when α-, β-, and γ_2-subunits were coexpressed in a single cell (Sigel et al., 1990; Burt and Kamatchi, 1991; Knoflach et al., 1992). GABA-gated chloride channels containing α-, β-, and γ-subunits, however, were not inhibited by Zn^{2+} (Draguhn et al., 1990).

In binding studies, the presence of binding sites for [^{3}H]muscimol, for [^{35}S]TBPS, and for [^{3}H]flunitrazepam was demonstrated on these receptors, and binding of these compounds to their binding sites could be modulated by other allosteric ligands of the $GABA_A$ receptor in a way similar to that found in the brain (table 1) (Pritchett et al., 1989; Pregenzer et al., 1993; Lüddens et al., 1994; Slany et al., 1994; Zezula et al., 1994). Thus, recombinant receptors containing α-, β-, and γ_2-subunits most closely resemble $GABA_A$ receptors found in the brain (Sigel et al., 1990; Verdoorn et al., 1990).

Depending on the subunit composition of recombinant receptors, various benzodiazepine receptor ligands exhibited a differential potency for inhibition of radiolabeled benzodiazepine binding. Data presently available are listed in tables 2, 3, 4, 5, 6, and 7. These studies indicated that the α- (table 2) and the γ-subunits (table 7) have the strongest influence on the affinity of the expressed $GABA_A$ receptors for benzodiazepine receptor ligands. Thus, most of the compounds listed in table 2 had similar affinities for recombinant receptors containing α_1-, α_2-, α_3-, or α_5-subunits. The affinities of the BZ_1 receptor-selective ligands 2-oxoquazepam, Cl 218872, β-CCM, or zolpidem, however, were four- to 17-fold higher for receptors containing α_1- than for receptors containing α_2- or α_3-subunits (table 2). Thus, receptors containing the α_1-subunit (together with an arbitrary β- and a γ_2-subunit) exhibited benzodiazepine binding properties corresponding to the BZ_1 receptor, whereas

TABLE 2

Comparison of benzodiazepine-binding properties of recombinant GABA$_A$ receptors consisting of β_2- and γ_2-, but different α-subunits

	Ki (nM) ± SEM					
	$\alpha_1\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_3\beta_2\gamma_2$	$\alpha_4\beta_2\gamma_2$	$\alpha_5\beta_2\gamma_2$	$\alpha_6\beta_2\gamma_2$
Benzodiazepines						
Diazepam	16.1 ± 1.0§,‖,¶,††	16.9 ± 5.5*,†	17.0 ± 1.8*,†	> 10,000§	14.9††	> 10,000§,¶
Clonazepam	1.3 ± 0.04¶	1.7 ± 0.4*,†	2.0 ± 0.04*,†	—	—	> 10,000¶
Flunitrazepam	2.0 ± 0.3§,‖,¶,††	3.3 ± 0.2‡	15.7 ± 3.6*,‖	> 10,000§	7.0 ± 0.3‡	> 10,000§,¶
Triazolam	1.8 ± 0.4*,‡,‖	1.2 ± 0.2*,‡	3.0 ± 0.7*,‖	—	1.2 ± 0.3*,‡,‖	> 10,000¶
Bretazenil	1.2 ± 0.4*,‡,‖	1.2 ± 0.2*,‡	1.3 ± 0.2*,‖	—	2.4 ± 0.5*,‡,‖	—
Ro15–1788	1.0 ± 0.1‡,§,¶,#	1.1 ± 0.04‡	1.5 ± 0.1#	107 ± 26§	0.4 ± 0.01‡	90 ± 20§,¶
Ro15–4513	10.4 ± 0.5‡,§,‖,¶	5.5 ± 0.1‡	7.8 ± 1.8*,‖	5.0 ± 0.9§	0.5 ± 0.04‡	5.1 ± 1.8§,¶
FG 8205	1.8 ± 0.1‡	3.8 ± 0.2‡	6.4 ± 2.3*,‖	—	3.7 ± 0.1‡	—
2-Oxoquazepam	20.0 ± 3.0¶	236 ± 12*,†	205 ± 18*,†	—	190 ± 15&,§§	> 10,000¶
Triazolopyridazines						
Cl 218872	130 ± 36*,†,‡‡	1820 ± 700*,†	1530 ± 290*,†	> 10,000§	490 ± 120&,§§	> 10,000§,¶
β-Carbolines						
β-CCM	1.7 ± 0.1‡,‖,¶	6.5 ± 0.6 ‡	4.1 ± 0.6*,†	—	27 ± 5&,§§	2050 ± 20¶
DMCM	5.0 ± 2¶	14.4 ± 7.0*,†	5.2 ± 0.2#	—	0.2††	210 ± 50¶
Abecarnil	3.9 ± 1.0*,‖	4.4 ± 0.6*,‖	7.1 ± 0.6*,‖	—	8.4 ± 0.1*,‖	—
Imidazopyridines						
Zolpidem	17.0 ± 1.2#,††	291 ± 10‡	357 ± 11#	—	> 15,000‡,††	—
Alpidem	9.2††	—	—	—	> 10,000††	—

* β_1 instead of β_2.
& β_3 instead of β_2.
Values are taken from the references indicated by the respective symbols:
† Pritchett et al., 1989.
‡ Hadingham et al., 1993b.
§ Wisden et al., 1991.
‖ Herb et al., 1992.
¶ Lüddens et al., 1990.
# Carter et al., 1992.
** Ymer et al., 1990.
†† Faure-Halley et al., 1993.
‡‡ Hadingham et al., 1992.
§§ Pritchett and Seeburg, 1990.
‖‖ Hadingham et al., 1993a.
¶¶ Lüddens et al., 1994.
The data in this table were selected to allow an extensive comparison of the binding properties of different recombinant receptors. Thus, when discrepant data were available, sets of data obtained from the same research group or using the same β-subunit in different recombinant receptors were selected to reduce differences in experimental conditions as far as possible. Other data indicating the variation of Ki values obtained by different authors are included in Tables 3–6.
FG 8205 = (7-chloro-5,6-dihydro-5-methyl-6-oxo-3(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazo-[1,5a][1,4]benzodiazepine.

receptors containing α_2- or α_3-subunits exhibited properties corresponding to BZ$_2$ benzodiazepine receptors (Pritchett et al., 1989; Pritchett and Seeburg, 1990).

Molecular biological studies have indicated that the exchange of a single amino acid (glycine for glutamic acid) at position 225 in the amino acid sequence of the α_3-subunit substantially increased the affinity of α_3-subunit-containing recombinant receptors for BZ$_1$ receptor-selective ligands (Pritchett and Seeburg, 1991). Thus, the type of a single amino acid present in this position of the α-subunit might largely determine the benzodiazepine binding properties of GABA$_A$ receptors. A slightly larger shift in the affinity for BZ$_1$ receptor-selective ligands resulted from changing an additional three adjacent residues (Pritchett and Seeburg, 1991). These amino acids therefore could be part of the benzodiazepine binding pocket of the GABA$_A$ receptors.

Interestingly, receptors containing the α_5-subunit together with an arbitrary β- and the γ_2-subunit (tables 2, 6) exhibited an extremely low affinity for the BZ$_1$ receptor-selective imidazopyridines zolpidem and alpidem (Pritchett and Seeburg, 1990) but have binding properties similar to BZ$_2$ receptors for 2-oxoquazepam. Thus, zolpidem is able to distinguish between GABA$_A$ receptors containing BZ$_1$- (high affinity), BZ$_2$- (intermediate affinity), or BZ$_3$ (extremely low affinity) receptors. The finding that Ro15–4513 and Cl 218872 exhibited a higher and β-CCM a lower affinity for receptors containing α_5-subunits than for receptors containing α_2- or α_3-subunits (table 2), might indicate that these compounds too exhibit distinct binding properties for α_5-subunit containing receptors. However, the variable Ki values for Cl 218872 or β-CCM obtained with α_5-subunit-containing receptors (table

TABLE 3

Comparison of benzodiazepine-binding properties of recombinant $GABA_A$ receptors consisting of α_1- and γ_2- but different β-subunits

	$\alpha_1\beta_1\gamma_2$	$\alpha_1\beta_2\gamma_2$	$\alpha_1\beta_3\gamma_2$
Benzodiazepines			
Diazepam	16.3 ± 0.5*	16.1 ± 1.0‡,§,‖,**	—
	59.7 ± 7.3††	10.3 ± 1.2¶	—
Clonazepam	1.3 ± 0.04*,#	1.3 ± 0.04‖	—
	—	4.8 ± 0.4¶	—
Flunitrazepam	2.0 ± 0.4#	2.0 ± 0.3‡,§,‖,**	3.1 ± 0.4‖ ‖
	11.5 ± 1.6†,††,§§	8.0 ± 0.2†	22.4 ± 2.5†
Triazolam	1.8 ± 0.4†,§§	0.8**	3.0 ± 0.9†
Bretazenil	1.2 ± 0.4†,§§	0.2**	1.2 ± 0.4†
Ro15–1788	0.5 ± 0.2*,†,#	1.0 ± 0.1†,‡,‖,¶	0.9 ± 0.04†,‡‡
Ro15–4513	10.0 ± 0.6†,§§	10.4 ± 0.5†,‡,§,‖	8.9 ± 0.9†
	5.0 ± 0.5††,‖ ‖		3.9 ± 0.8‖ ‖
FG 8205	2.3 ± 0.5†,††,§§	1.8 ± 0.1†	2.3 ± 0.5†
2-Oxoquazepam	19.6 ± 2.5*,#	20.0 ± 3.0§,‖	16.3 ± 1.9‡‡,‖ ‖
Triazolopyridazines			
Cl 218872	130 ± 36*,††	130 ± 40‡,‖	120 ± 18‡‡
	290 ± 32†,§§	220 ± 37†	301 ± 28†
		73 ± 6¶,**	
β-Carbolines			
β-CCM	0.8 ± 0.1*,††,§§	1.7 ± 0.1†,§,‖	0.8 ± 0.2‡‡
	2.2 ± 0.4†		3.8 ± 1.1†
β-CCE	—	0.3 ± 0.01¶	—
DMCM	5.3 ± 1.5*,#	5.0 ± 2.0‖	—
	27.1 ± 5.6††	2.6 ± 0.3¶,**	—
Abecarnil	3.9 ± 1.0§§	—	—
FG 7142	—	16.7 ± 2.5¶	—
Imidazopyridines			
Zolpidem	112 ± 17†,§§	60 ± 20†	64 ± 8.0†
	54.2 ± 2.0††	17 ± 1.2¶,**	19 ± 3.5‡‡
		30§	
Alpidem	—	9.2**	—
Cyclopyrrolones			
Zopiclone	—	56**	—
Suriclone	—	0.2 ± 0.05¶,**	—

Values (Ki (nM) ± SEM) are taken from the references indicated by the respective symbols. When significantly different Ki values were obtained by different authors, they are listed separately. A direct comparison of Ki values can only be made with data from the same authors.

* Pritchett et al., 1989.
† Hadingham et al., 1993b.
‡ Wisden et al., 1991.
§ Herb et al., 1992.
‖ Lüddens et al., 1990.
¶ Carter et al., 1992.
# Ymer et al., 1990.
** Faure-Halley et al., 1993.
†† Hadingham et al., 1992.
‡‡ Pritchett and Seeburg, 1990.
§§ Hadingham et al., 1993a.
‖ ‖ Lüddens et al., 1994.

6) indicate, that more extensive investigations are necessary to support this conclusion.

Other studies indicated that receptors containing α_4- or α_6-subunits together with β_2- and γ_2-subunits exhibited a high affinity for GABA and muscimol and for the partial inverse agonist Ro15–4513 but a low affinity for the benzodiazepine receptor antagonists Ro15–1788. Receptors consisting of $\alpha_6\beta_2\gamma_2$ subunits additionally exhibited a low affinity for the β-carbolines β-CCM or DMCM (table 2). Both of these receptors, however, exhibited no affinity at all for Cl 218872 or other benzodiazepine binding site agonists (Lüddens et al., 1990; Wisden et al., 1991). Thus, Ro15–4513, in contrast to all other benzodiazepine binding site ligands, exhibits high affinity for all $GABA_A$ receptors consisting of $\alpha\beta\gamma$ subunits and is the most versatile benzodiazepine binding site ligand presently available. These binding studies were supported by electrophysiological experiments indicating that GABA-induced Cl^- ion flux in receptors containing α_6-subunits could not be modulated by flunitrazepam (Kleingoor et al., 1991).

Recent studies have indicated that the molecular reason for the lack of modulation by benzodiazepine binding site agonists of α_6-subunit-containing receptors might

TABLE 4

*Comparison of benzodiazepine-binding properties of recombinant GABA$_A$ receptors consisting of α_2- and γ_2- but different β-subunits**

	$\alpha_2\beta_1\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_2\beta_3\gamma_2$
Benzodiazepines			
Diazepam	16.9 ± 5.5†	—	—
Clonazepam	1.7 ± 0.4†	—	—
Flunitrazepam	5.2 ± 0.4‡,‖ ‖	3.3 ± 0.2‡	9.6 ± 1.6‡
Triazolam	1.2 ± 0.2‡,‖ ‖	—	1.9 ± 0.3‡
Bretazenil	1.2 ± 0.2‡,‖ ‖	—	2.1 ± 0.2‡
Ro15–1788	0.9 ± 0.1†,‡	1.1 ± 0.04‡	1.1 ± 0.05‡,§§
Ro15–4513	10.4 ± 1.1‡,‖ ‖	5.5 ± 0.1‡	8.1 ± 2.5‡
FG 8205	3.7 ± 0.2‡,‖ ‖	3.8 ± 0.2‡	6.6 ± 1.9‡
2-Oxoquazepam	236 ± 12†	—	225.0 ± 12§§
Triazolopyridazines			
Cl 218872	2903 ± 420‡,‖ ‖	1058 ± 211‡	3470 ± 903‡
	1820 ± 700†	—	1786 ± 620§§
β-Carbolines			
β-CCM	6.5 ± 1.2‡,‖ ‖	6.5 ± 0.6‡	15.7 ± 1.1‡
	2.9 ± 0.4†	—	3.4 ± 0.4§§
DMCM	14.4 ± 7.0†	—	—
Abecarnil	4.4 ± 0.6‖ ‖	—	—
Imidazopyridines			
Zolpidem	761 ± 88‡,‖ ‖	291.4 ± 10.1‡	427.0 ± 31.2‡,§§

* Ki (nM) ± SEM.

Values were taken from the references indicated by the respective symbols. When significantly different Ki values were obtained by different authors, they were listed separately. A direct comparison of Ki values can only be made with data from the same authors.

† Pritchett et al., 1989.
‡ Hadingham et al., 1993b.
§ Wisden et al., 1991.
‖ Herb et al., 1992.
¶ Lüddens et al., 1990.
# Carter et al., 1992.
** Ymer et al., 1990.
†† Faure-Halley et al., 1993.
‡‡ Hadingham et al., 1992.
§§ Pritchett and Seeburg, 1990.
‖ ‖ Hadingham et al., 1993a.
¶¶ Lüddens et al., 1994.

be a single arginine residue in the position 100 of the amino acid sequence of the α_6-subunit. In the homologous position 101 of the α_1-subunit, a histidine is present instead of the arginine. (Wieland et al., 1992). If this histidine, which is present not only in the α_1- but also in the corresponding position of the α_2-, α_3-, and α_5-subunits, is replaced by an arginine, the high affinity binding of benzodiazepine agonists is lost in the respective recombinant receptor. This histidine thus seems to be a key residue for the action of clinically used benzodiazepine ligands (Kleingoor et al., 1993).

Most of the information so far available indicates that the type of β-subunit only slightly influenced the benzodiazepine binding properties of recombinant receptors (tables 3–6). Recently, however, it was demonstrated that the β_1-, β_2-, or β_3-subunits differentially influenced the [^{35}S]TBPS binding of recombinant receptors and its modulation by benzodiazepines (Lüddens et al., 1994). In addition, it was demonstrated that the action of the anticonvulsant loreclezole depended on the type of the β-subunit present in recombinant receptors. This compound potentiated the action of GABA but exhibited a more than 300-fold higher affinity for receptors containing β_2- or β_3-subunits over those containing β_1-subunits (Wingrove et al., 1994). The investigation of the properties of recombinant GABA$_A$ receptors containing mutated chimeric β_1/β_2 GABA$_A$ receptor subunits allowed the identification of a single amino acid located at the carboxyl-terminal end of the putative channel-lining transmembrane domain TM$_2$, which confers sensitivity to the modulatory effects of loreclezole (Wingrove et al., 1994).

Most of the studies so far performed have used the short (γ_{2S}-) form of the γ_2-subunit for the construction of recombinant receptors. Recent evidence seems to indicate that GABA$_A$ receptors containing the alternatively spliced long (γ_{2L}-) form of the γ_2-subunit, in contrast to those containing the γ_{2S}-subunit, could be modulated by ethanol (Wafford et al., 1991). This, however, was questioned by other studies that could not observe a differential response to ethanol in subunit combinations containing different γ_2-subunit splice variants (Sigel et al.,

TABLE 5

*Comparison of benzodiazepine-binding properties of recombinant $GABA_A$ receptors consisting of α_3- and γ_2- but different β-subunits**

	$\alpha_3\beta_1\gamma_2$	$\alpha_3\beta_2\gamma_2$	$\alpha_3\beta_3\gamma_2$
Benzodiazepines			
Diazepam	17.0 ± 1.8†	8.6 ± 1.1‡	—
Clonazepam	2.0 ± 0.04†	4.2 ± 0.5‡	—
Flunitrazepam	15.7 ± 3.6‖	—	—
Triazolam	3.0 ± 0.7‖	—	—
Bretazenil	1.3 ± 0.2‖	—	—
Ro15–1788	0.7 ± 0.2†	1.5 ± 0.1‡	0.6 ± 0.2§
Ro15–4513	7.8 ± 1.8‖	—	—
FG 8205	6.4 ± 2.3‖	—	—
2-Oxoquazepam	205 ± 18†	—	201 ± 18§
Triazolopyridazines			
Cl 218872	1530 ± 290†	549 ± 61‡	1495 ± 230§
	3136 ± 600‖	—	—
β-Carbolines			
β-CCM	4.1 ± 0.6†	—	4.1 ± 0.6§
	9.2 ± 1.8‖		
β-CCE	—	6.5 ± 1.1‡	—
DMCM	10.6 ± 6.3†	5.2 ± 0.2‡	—
Abecarnil	7.1 ± 0.6‖	—	—
FG 7142	—	74.9 ± 14‡	—
Imidazopyridines			
Zolpidem	2150 ± 492‖	357 ± 11‡	398 ± 43§
Cyclopyrrolones			
Suriclone	—	0.3 ± 0.01‡	—

* Ki (nM) ± SEM.

Values were taken from the references indicated by the respective symbols. When significantly different Ki values were obtained by different authors, they were listed separately. A direct comparison of Ki values can only be made with data from the same authors.

† Pritchett et al., 1989.

‡ Carter et al., 1992.

§ Pritchett and Seeburg, 1990.

‖ Hadingham et al., 1993a.

1993; Ryan-Jastrow and Macdonald, 1993). These results and the observation that the long version of the γ_2-subunit is present in brain regions where ethanol did not affect GABA function (Glencorse et al., 1992) suggest that the presence of the long variant of the γ_2-subunit alone is not sufficient for ethanol's action to enhance responses to GABA (Criswell et al., 1993).

The γ_{2L}-$GABA_A$ receptor subunit, however, has been shown previously to contain a consensus phosphorylation sequence for protein kinase C (Whiting et al., 1990), and in vitro mutagenesis and expression in *Xenopus* oocytes has demonstrated that this consensus site contained in the γ_{2L}-insert is critical for modulation of $GABA_A$ receptors by ethanol. In addition, inhibition of protein kinase C could prevent ethanol enhancement of GABA-induced Cl^- ion flux (Wafford and Whiting, 1992; Weiner et al., 1994). It is thus possible that phosphorylation or dephosphorylation of a specific site on the $GABA_A$ receptor protein can act as a control mechanism for neuronal responses to alcohol exposure. These observations and the finding of a differential localization of the two alternatively spliced $GABA_A$ receptor γ_2-subunits in the brain (Glencorse et al., 1992; Miralles et al., 1994) might explain discrepant results indicating an enhancement of GABAergic function by ethanol in some, but not all, species and brain tissues investigated (Ticku, 1990).

Receptors containing a γ_1- instead of a γ_2-subunit exhibited a reduced affinity for benzodiazepines and no affinity for the benzodiazepine receptor antagonist Ro15–1788 (table 7). In addition, most but not all of the benzodiazepine receptor agonists investigated exhibited a reduced efficacy for the enhancement of GABAergic transmission in receptors containing γ_1-subunits as compared with γ_2-subunit-containing receptors (Puia et al., 1991, 1992; Ducic et al., 1993; Giusti et al., 1993). The β-carboline DMCM, which exhibited inverse benzodiazepine receptor agonist properties in γ_2-subunit-containing receptors, even exhibited partial benzodiazepine receptor agonist properties in receptors containing γ_1-subunits (Puia et al., 1991). These data, and the finding that astrocytes express the γ_1-subunit gene to higher levels than those of other γ-subunits (Bovolin et al., 1992), provide an explanation for the previous observation that DMCM functions as an agonist on $GABA_A$ receptors of astrocytes (Bormann and Kettenmann, 1988; Rosewater and Sontheimer, 1994).

These observations were extended to other $GABA_A$ receptor subunit combinations, and evidence accumulated indicates that the efficacy of benzodiazepine receptor ligands to enhance or reduce GABAergic transmission changes with the type of α-, β-, or γ-subunit present in the recombinant receptors investigated (Von Blankenfeld et al., 1990; Kleingoor et al., 1991; Puia et al., 1991, 1992; Wafford et al., 1993a, b; Ducic et al., 1993; Giusti et al., 1993; Im et al., 1993b, c; Knoflach et al., 1993). Thus, a compound could act as a full agonist at one and as a partial agonist at another receptor.

However, the efficacy for enhancement of GABA-induced chloride ion flux is extremely dependent on the exact experimental conditions (electrode buffer, holding potential, way and time course of application of GABA and drugs) used. In addition, it depends on the efficacy of GABA for opening Cl^--channels at the particular receptor investigated and on the exact GABA- and drug-concentration applied. Thus, even when the same experimental conditions are used for the investigation of different recombinant receptors, a careful GABA-dose-response curve must be established for each recombinant receptor, before—after chosing a GABA-concentration equivalent for the different receptors—the drug-dose-response curve can be measured. Because these conditions in most of the studies mentioned above have not been met, a quantitative comparison of the efficacies of different benzodiazepine binding site ligands for enhancement of GABA-induced chloride ion flux in different recombinant receptors is presently not possible.

Receptors containing a γ_3- instead of a γ_2-subunit exhibited a high affinity for the benzodiazepine binding site antagonist Ro15–1788 and the partial inverse ago-

TABLE 6

*Comparison of benzodiazepine-binding properties of recombinant GABA$_A$ receptors consisting of α_5- and γ_2- but different β-subunits**

	$\alpha_5\beta_1\gamma_2$	$\alpha_5\beta_2\gamma_2$	$\alpha_5\beta_3\gamma_2$
Benzodiazepines			
Diazepam	—	14.9‡	17 ± 2.0¶
Flunitrazepam	5.5 ± 0.4†,‖	7.0 ± 0.3†	12.1 ± 1.3†
	—	1.2‡	2.1 ± 0.2¶
Triazolam	1.2 ± 0.3†,‖	0.8‡	2.7 ± 0.2†
Alprazolam	—	4.8‡	—
Midazolam	—	0.9‡	—
Bretazenil	2.4 ± 0.5†,‖	0.5‡	2.1 ± 0.5†
Ro15–1788	0.6 ± 0.03†	0.4 ± 0.01†,‡	0.5 ± 0.04†,¶
Ro15–4513	0.7 ± 0.1†,‖	0.5 ± 0.04†	1.0 ± 0.2†
			0.4 ± 0.03¶
FG 8205	6.4 ± 0.03†,‖	3.7 ± 0.1†	5.9 ± 1.2†
2-Oxoquazepam	—	—	190 ± 15§
			122 ± 24¶
Triazolopyridazines			
Cl 218872	1154 ± 66†,‖	2624 ± 200†	1835 ± 803†
	—	344‡	490 ± 120§
			280 ± 14¶
β-Carbolines			
β-CCM	76.4 ± 7.8†,‖	125 ± 1.8†	260 ± 51†
	—	—	27.0 ± 5§
Abecarnil	8.4 ± 0.1‖	—	—
Imidazopyridines			
Zolpidem	>15,000†,‖	>15,000†,‡	>15,000†,§,¶
Alpidem	—	>10,000‡	—
Cyclopyrrolones			
Zopiclone	—	64.4‡	—
Suriclone	—	0.2‡	—

* Ki (nM) ± SEM.

Values were taken from the references indicated by the respective symbols. When significantly different Ki values were obtained by different authors, they were listed separately. A direct comparison of Ki values can only be made with data from the same authors.

† Hadingham et al., 1993b.

‡ Faure-Halley et al., 1993.

§ Pritchett and Seeburg, 1990.

‖ Hadingham et al., 1993a.

¶ Lüddens et al., 1994.

nist Ro15–4513 but a significantly reduced affinity for benzodiazepine agonists (table 7). In addition, these receptors exhibited a high affinity for Cl 218872 and a very low affinity for zolpidem (Herb et al., 1992; Lüddens et al., 1994). Similarly, receptors containing $\alpha_5\beta_3\gamma_3$ subunits exhibited a high affinity for Ro15–1788, Ro15–4513, and Cl 218872, a reduced affinity for benzodiazepine agonists, and a very low affinity for zolpidem (data not shown) (Lüddens et al., 1994). Thus, a low affinity for zolpidem not only is caused by the α_5- but also by the γ_3-subunit of GABA$_A$ receptors.

No data are available on the benzodiazepine binding properties of GABA$_A$ receptors containing the δ-instead of the γ_2-subunit. However, in the original report on this subunit, preliminary experiments are mentioned indicating that a triple subunit combination containing δ-instead of γ_2-subunits do not seem to form high affinity benzodiazepine binding sites (Shivers et al., 1989). GABA$_A$ receptors that cannot be modulated by benzodiazepines have been known for many years and seem to be enriched in cerebellum (see section II.O. of this article). The relative enrichment of δ-subunits in cerebellum (Shivers et al., 1989; Benke et al., 1991a; Persohn et al., 1992) may indicate that a subunit combination containing δ-subunits could be the molecular basis for GABA$_A$ receptors not modulated by benzodiazepines. It has to be stressed, however, that combinations containing only α- and β-, but no γ-subunits (Pritchett et al., 1989; Sigel et al., 1990), or receptors containing either α_4- (Wisden et al., 1991) or α_6-subunits (Lüddens et al., 1990), also seem to produce GABA$_A$ receptors that cannot be modulated by benzodiazepine agonists.

5. Properties of receptors consisting of more than three different subunits. Recombinant receptors produced by a combination of three different subunits most closely resemble GABA$_A$ receptors found in the brain. This might have been one of the reasons why recombinant receptors containing more than three different subunits only have been investigated in two reports (Sigel et al., 1990; Verdoorn, 1994). At least some of these receptors seem to assemble and exhibit conductance and activation properties that are different from those of receptors containing only three different subunits. It has to be kept in mind, however, that receptors containing four or five

TABLE 7

Comparison of benzodiazepine-binding properties of recombinant $GABA_A$ receptors consisting of α_1-, β_1/β_2- but different γ-subunits†

	$\alpha_1\beta_1\gamma_1$	$\alpha_1\beta_2\gamma_2$	$\alpha_1\beta_2\gamma_3$
Benzodiazepines			
Diazepam	—	16.3 ± 0.5§,‖,¶,††	670‖
	—	—	308 ± 116*,§§
Clonazepam	320 ± 60**	1.3 ± 0.04¶	—
Flunitrazepam	20 ± 5**	2.0 ± 0.3§,‖,¶,††	220‖
	—	3.1 ± 0.4*,§§	67 ± 4.0*,§§
Midazolam	—	1.5‖,††	27‖
Ro15–1788	>10,000**	1.0 ± 0.1‡,§,¶,#	2.0 ± 0.2‖
	—	—	0.9 ± 0.3*,§§
Ro15–4513	—	10.4 ± 0.5‡,§,‖,¶	5.5‖
	—	3.9 ± 0.8*,§§	2.8 ± 0.9*,§§
2-Oxoquazepam	380 ± 52**	20 ± 3¶	540‖
	—	16 ± 2*§§	210 ± 23*,§§
Triazolopyridazines			
Cl 218872	—	130 ± 40§,¶,*§§	8.0 ± 1.0*,§§
β-Carbolines			
β-CCM	—	1.7 ± 0.1‡,‖,¶	5.4‖
DMCM	>10,000**	5.0 ± 2¶	—
	—	2.6 ± 0.3#,††	—
Imidazopyridines			
Zolpidem	—	17.0 ± 1.2#,††,*,§§	>10,000*,§§
	—	30‖	5500‖

* β_3 instead of β_2; Values were taken from the references indicated by the respective symbols. When significantly different Ki values were obtained by different authors, they were listed separately. A direct comparison of Ki values can only be made with data from the same authors.

† Ki (nM) ± SEM.
‡ Hadingham et al., 1993b.
§ Wisden et al., 1991.
‖ Herb et al., 1992.
¶ Lüddens et al., 1990.
# Carter et al., 1992.
** Ymer et al., 1990.
†† Faure-Halley et al., 1993.
§§ Lüddens et al., 1994.

different subunits can only be adequately investigated and distinguished from a mixture of different receptors consisting of three subunits in the single channel recording mode of the patch clamp technique.

6. Properties of receptors containing ρ-subunits. The highest concentration of mRNA for ρ_1- and ρ_2-subunits has been found in the retina. The additional presence of these subunits in bovine cerebellum and cerebral cortex has, however, been demonstrated by Northern blot analysis (Cutting et al., 1991). Expression of human ρ_1-subunits in *Xenopus* oocytes resulted in the formation of homo-oligomeric chloride channels that displayed large GABA-gated currents (Cutting et al., 1991; Shimada et al., 1992). Currents from ρ_1-GABA channels, compared with $\alpha_1\beta_2\gamma_2$ GABA channels, were 40-fold more sensitive to GABA, activated eight-fold more slowly at a GABA concentration required to activate half the current, did not desensitize with maintained agonist-application, and closed eight-fold more slowly after agonist removal (Amin and Weiss, 1994). The GABA-induced Cl^- ion flux of homo-oligomeric receptors containing the ρ_1- or ρ_2-subunit could be inhibited by picrotoxin but not by bicuculline (Cutting et al., 1991, 1992; Kusama et al., 1993a, b). In addition, this GABA effect was not modulated by pentobarbital, by the inhalation anesthetic isoflurane, or by benzodiazepine receptor ligands (table 1) (Kusama et al., 1993a; Harrison et al., 1993), and the pharmacology of these channels was not altered by coexpression with α-, β-, or γ-subunits (Cutting et al., 1991; Shimada et al., 1992). In addition, recombinant receptors containing ρ-subunits were insensitive to the $GABA_B$ receptor agonist baclofen (Cutting et al., 1991; Shimada et al., 1992). Because ρ-subunit-containing receptors are insensitive to bicuculline and baclofen, they partially resemble $GABA_C$ receptors (Johnston 1986; Silvilotti and Nistri, 1991; Shimada et al., 1992) (see section I. of this article). Future investigations will have to clarify whether all properties of $GABA_C$ receptors can be reproduced by ρ-subunit-containing receptors.

Pharmacological features similar to those of recombinant receptors containing ρ-subunits have been identified in *Xenopus* oocytes expressing mRNA extracted from whole retina (Polenzani et al., 1991; Kusama et al., 1993a; Woodward et al., 1992, 1993).

7. Comments on the pharmacology of recombinant γ-aminobutyric $acid_A$ receptors. The investigation of the

structure, pharmacology, and electrophysiology of recombinant GABA$_A$ receptors is essential for the understanding of GABA$_A$ receptors in the brain. In order to obtain consistent and meaningful results, however, several precautions have to be taken.

Recently, the expression of low levels of β_3-subunit mRNA in untransfected HEK 293 cells has been demonstrated (Kirkness and Fraser, 1993). Owing to the widespread use of these cells for expression of recombinant GABA$_A$ receptors, even a low level of endogenous β_3-subunit expression has significant implications. Endogenously expressed subunits possibly can combine with subunits exogenously introduced into these cells and influence the properties of recombinant receptors. On the other hand, the exogenously introduced subunit mRNA probably is much more abundant than the endogenous β_3-mRNA. Exogenous and endogenous subunits do not necessarily combine; if they do, the resulting subunit combinations probably are not very abundant (Fuchs et al., 1995). This conclusion is in agreement with a recent report indicating that 13 cell lines derived from diverse tissue origins, although containing detectable GABA$_A$ receptor subunit mRNAs, in most cases did not exhibit GABA-evoked currents in the whole cell configuration of the patch clamp technique (Hales and Tyndale, 1994). In any case, the possible contribution of endogenous GABA$_A$ receptor subunits to the function of recombinant receptors has to be thoroughly investigated for every cell line used for expression of these receptors.

Several recent studies have indicated that the exchange of a single amino acid in a GABA$_A$ receptor subunit could dramatically influence the properties of recombinant GABA$_A$ receptors produced with this subunit (Pritchett and Seeburg, 1991; Sigel et al., 1992; Wieland et al., 1992; Korpi et al., 1993). In at least two cases (Sigel et al., 1992; Angelotti et al., 1992), allelic variants of GABA$_A$ receptor subunits have been cloned with single amino acid substitutions. In one of these cases, the use of the allelic variant caused a dramatic alteration of the properties of the resulting recombinant GABA$_A$ receptors and led to wrong conclusions on the properties of receptors containing this subunit (Sigel et al., 1990, 1992). This should be a warning not to use GABA$_A$ receptor subunits with an amino acid sequence not identical to the published sequence for transfection studies without a thorough investigation of the consequences of the mutation (Angelotti et al., 1992; Sigel et al., 1992).

In addition, the actual expression in the cells of the subunits used for transfection must be thoroughly checked. In at least one case, it could be demonstrated that although cells were stably transfected with a triple subunit combination, these cells actually expressed only two subunits (Wong et al., 1992). Failure to check for the actual expression of subunits could lead to an erroneous attribution of pharmacological properties to a receptor subtype that never was expressed in the cells during the respective experiments.

Furthermore, it must be kept in mind that even when the cells correctly express all the subunits used for transfection, depending on the subunit stoichiometry and on the arrangement of subunits within the receptor complex, a variety of structurally different receptors possibly could form. Thus, cells making only two subunits (A, B) could theoretically form receptors in as many as eight different arrangements (A_5, A_4B, two different arrangements of A_3B_2 or A_2B_3, AB_4 and B_5). Cells containing three subunits could form up to 51 receptors with different subunit arrangements (for review, see Burt and Kamatchi, 1991). No information on the possible functional consequences of different subunit stoichiometries or of different subunit arrangements in receptors with the same subunit stoichiometry is available, but it can be assumed that receptors with different structure will have different properties.

Results presently available indicate that certain subunit combinations are preferred intermediates during the assembly process and that, possibly, a single preferred configuration of GABA$_A$ receptors may exist (Verdoorn et al., 1990; Knoflach et al., 1992; Angelotti et al., 1993a; Angelotti and Macdonald, 1993) (see section IV.E. of this article). Recently, however, it has been demonstrated that there were clear differences in the sensitivity of GABA responses to bicuculline and pentobarbital when the same subunit combination was expressed in *Xenopus* oocytes or in Chinese hamster ovary cells (Valeyev et al., 1993). Although a different post-translational modification of receptors in the two systems might have been the cause of this discrepancy, the differential ability of these cell systems to express receptors consisting of $\alpha\beta$, $\beta\gamma$, or $\alpha\gamma$ subunits (see section III.B.3) indicates that *Xenopus* oocytes and certain cell lines might have different ways to assemble these receptors (Angelotti et al., 1993a). Recently, it has been demonstrated that the assembly of the nicotinic acetylcholine receptor is stimulated by the phosphorylation of its γ-subunit (Green et al., 1991; Ross et al., 1991). A similar mechanism might be active with GABA$_A$ receptors (Angelotti et al., 1993b) and could thus allow the cells to modulate the composition and properties of these receptors. Such regulatory mechanisms might be one cause of the plasticity of GABA$_A$ receptors in the brain (see section V. of this article).

Another factor that could influence the assembly of recombinant receptors is the genetic information provided to the cells. Thus, the absence or presence of 5′ or 3′ regulatory sequences on the GABA$_A$ receptor subunit mRNA or cDNA constructs used to inject *Xenopus* oocytes or to transfect cells in culture, respectively, might influence the stability of mRNA (Jackson, 1993) and, thus, the efficiency of expression of GABA$_A$ receptor subunits. Similarly, the type of the vector used for transfection or the mode of transfection of cells might influ-

ence the efficiency of expression of subunits. Thus, depending on the strength of the promoter present in the vector used for transfection, the cells will synthesize more or fewer of the $GABA_A$ receptor subunits. The use of a virus as a vehicle for transfection (baculovirus system) is not only much more effective than other transfection methods, but, because of the massive production of virus particles in the infected cells, a large amount of $GABA_A$ receptor subunits will be synthesized. The concentration of $GABA_A$ receptor subunits synthesized within the cells certainly will influence the stoichiometry of $GABA_A$ receptors formed. A low concentration of subunits will favor the assembly of subunits with a high affinity for each other, whereas a high concentration of subunits may lead to inappropriate subunit association.

Thus, the composition (subunit stoichiometry and arrangement) and properties of recombinant receptors produced from the same subunits in different cell systems might be different, depending on the model system used, and might be different from that of receptors found in the brain. This could be one of the reasons for the variability in the Ki values for the same benzodiazepine binding site ligands, observed by comparing data from different authors (tables 3–7). It is to be expected that a homologous expression system (for instance, neurons expressing their endogenous $GABA_A$ receptors) will provide more valid information on the properties of these receptors in the brain than a heterologous system, using non-neuronal cells and $GABA_A$ receptor mRNAs or cDNAs from a different species. Nevertheless, heterologous expression studies of $GABA_A$ receptors have clearly demonstrated the potential that exists for the generation of $GABA_A$ receptor heterogeneity and have revealed valuable information on the different contributions that subunits can make to a receptors pharmacological profile. However, so far, neither the subunit stoichiometry nor the subunit arrangement within receptors has been determined in recombinant or in native receptors.

Related to the problem of subunit arrangement within a $GABA_A$ receptor is the question of whether receptors consisting of two subunits coexist with receptors containing three different subunits after transfection of cells with a triple subunit combination. Although there are studies indicating that $GABA_A$ receptors consisting of only α- and β-subunits were rarely if ever formed upon co-expression of α-, β-, and γ-subunits (Angelotti and Macdonald, 1993; Angelotti et al., 1993a) and that the $\alpha\beta\gamma$- $GABA_A$ receptors were the preferred final form of the receptor channels, the simultaneous sensitivity of the GABA response of dorsal root ganglion neurons to Zn^{2+} and La^{3+} (Ma and Narahashi, 1993a) and the finding that the GABA response of many neurons in the whole cell recording mode is both positively modulated by benzodiazepines but negatively modulated by Zn^{2+} (Celentano et al., 1991) support the conclusion of a possible coexistence of $\alpha\beta$- and $\alpha\beta\gamma$-receptors in the same cell. It has to be assumed, therefore, that at least certain cells have the possibility of simultaneously or subsequently assembling $GABA_A$ receptors consisting of $\alpha\beta$- or $\alpha\beta\gamma$-subunits.

Finally, the function of $GABA_A$ receptors could be modulated by the native environment of the neuron, such as membrane lipid composition (Koenig and Martin, 1992), endogenous protein factors, interacting modulatory kinases or phosphatases (Stelzer, 1992) (see section V.D. of this article), or could be influenced by post-translational modification of receptor proteins. All these factors must be considered when comparing properties of $GABA_A$ receptors derived from expressed cDNA to those found in the central nervous system.

So far, only a few subunit combinations have been used in the course of investigating the properties of recombinant receptors. In addition, the actual presence of allosteric modulatory sites and their pharmacology on the resulting recombinant receptors has not been systematically investigated. Thus, the investigation of the pharmacology of recombinant receptors is just beginning.

IV. Structure of γ-Aminobutyric $Acid_A$ Receptor Subtypes in the Brain

Results obtained so far indicate that receptors with different subunit composition exhibit different pharmacological and electrophysiological properties. It has been estimated that, depending on the number and types of different subunits forming the $GABA_A$ receptor, up to several hundred subunit combinations and more than 150,000 different subunit arrangements are possible (Burt and Kamatchi, 1991). Not all of these subunit combinations and arrangements can reasonably be investigated. But a large part of these possible combinations might not even exist in the brain and each subunit combination might have a single preferred subunit arrangement. The identification of those subunit combinations actually occurring in the brain is therefore not only of theoretical but also of tremendous practical importance.

A. Regional Distribution of γ-Aminobutyric $Acid_A$ Receptor Subunit mRNAs in the Brain

"In situ" hybridization studies have indicated that the mRNAs of the various subunits display a distinct but overlapping regional distribution in the brain (Wisden et al., 1992; Persohn et al., 1992; Laurie et al., 1992a; Miralles et al., 1994). Whereas the mRNA of the α_1-subunit is widely distributed, other mRNAs have a more restricted distribution. The mRNA of the α_6-subunit, however, so far has been identified only in cerebellar and cochlear granule cells (Wisden et al., 1992; Laurie et al., 1992a; Drescher et al., 1993; Varecka et al., 1994). Some neuronal populations co-express large numbers of subunit mRNAs, whereas in others, only a few $GABA_A$ receptor-specific mRNAs are found. Neocortex, hippocampus, and caudate/putamen display complex ex-

pression patterns, and these areas probably contain a large diversity of GABA$_A$ receptors (Persohn et al., 1992; Wisden et al., 1992; Laurie et al., 1992a).

In many brain regions, a consistent co-expression is observed for α_1-, β_2-, and γ_2-mRNAs. Colocalization is also apparent for the α_2- and β_3- mRNAs, and these predominate in areas such as amygdala and hypothalamus. In much of the forebrain, with the exception of the hippocampal pyramidal cells, the α_4- and δ-transcripts seem to codistribute. The α_5- and β_1-mRNAs may encode predominantly hippocampal forms of the GABA$_A$ receptor (Wisden et al., 1992). Because γ_1- and γ_3-subunit transcripts are found in a number of specific brain regions, albeit at lower levels than that of the γ_2-subunit (Laurie et al., 1992a; Wisden et al., 1992), it is possible that receptors containing these α- and β-, or α- and δ-subunit combinations may additionally contain γ_1-, γ_2- or γ_3-subunits. There are a few nuclei that contain lower levels of the γ_2-subunit mRNA relative to that of the γ_1-subunit (e.g., medial amygdaloid nucleus) or the γ_3-subunit (e.g., medial geniculate nucleus) transcripts (Wisden et al., 1992). In these regions, however, assignment of subunit combinations is difficult because of the large number of other transcripts present. Recently, evidence has accumulated for a differential distribution of the alternatively spliced γ_2-forms in the brain. Thus, there are brain regions or neuronal types expressing either the γ_{2L}-, the γ_{2S}, or each of these two transcripts (Glencorse et al., 1992; Miralles et al., 1994).

In yet other brain tissues, γ-subunits seem not to occur. Thus, in thalamus, a region that has been proposed to contain GABA$_A$ receptors without associated benzodiazepine binding sites (Olsen et al., 1990), the only abundant GABA$_A$ receptor mRNAs are those of α_1, α_4, β_2, and, to a lesser extent, δ. It is thus possible that $\alpha_1\alpha_4\beta_2$ occur together with the addition of δ in some thalamic nuclei (Wisden et al., 1992). Cerebellar granule cells express significant quantities of α_1-, α_6-, β_2-, β_3-, γ_2-, and δ-mRNAs, whereas Purkinje cells seem to contain only the α_1-, β_2-, β_3-, and γ_2-mRNAs (Persohn et al., 1992; Laurie et al., 1992a). GABA$_A$ receptor subunits were also found on a few non-neuronal cells, including adrenal chromaffin cells (Bormann and Clapham, 1985), astrocytes (Bender and Hertz, 1987; Bormann and Kettenman, 1988; Bovolin et al., 1992), and Bergmann glia cells (Laurie et al., 1992a). In addition, GABA$_A$ receptors have been identified in several peripheral tissues (Erdö and Wolff, 1990; Bertrand and Galligan, 1992). Putative Bergmann glia, found in the Purkinje cell layer of the cerebellum, seem to contain only α_2- and γ_1- subunit transcripts (Laurie et al., 1992a), a combination that has not yet been extensively investigated.

Each transcript exhibits a unique regional and age-specific developmental expression profile (Laurie et al., 1992b; Poulter et al., 1992, 1993; Ma et al., 1993), indicating that the composition, and presumably the properties, of embryonic and early postnatal rat GABA$_A$ receptors differ markedly from those expressed in adult brain.

However, the apparent absence of a particular subunit mRNA in a given area may be caused by a low expression of the corresponding gene or to mRNA instability. In addition, the mRNA expression possibly could change, not only during development, but also in the course of adaptation to environmental stimuli (Bovolin et al., 1992). The presence of mRNA for a given subunit also does not necessarily mean it is expressed to form a functional receptor and the amount of protein subunit expressed does not necessarily correlate with the amount of mRNA present in the cell (Bovolin et al., 1992; Williamson and Pritchett, 1994). The "in situ" hybridization studies thus must be supplemented by immunohistochemical studies using antibodies directed against the various GABA$_A$ receptor subunits (see section IV.C. of this article).

B. Biochemical, Pharmacological, and Immunological Identification of γ-Aminobutyric Acid$_A$ Receptor Subunit Proteins

Biochemical studies supported a molecular heterogeneity of GABA$_A$ receptors long before such a heterogeneity was substantiated by molecular biological studies. In 1980, it was demonstrated that the benzodiazepines [^{3}H]flunitrazepam (Möhler et al., 1980; Sieghart and Karobath, 1980) or [^{3}H]clonazepam (Sieghart and Möhler, 1982), can be used as photoaffinity ligands for the benzodiazepine binding site of the GABA$_A$ receptor. In photolabeling experiments, [^{3}H]flunitrazepam irreversibly labeled predominantly a single protein with an apparent molecular weight of 51 kDa (P_{51}) in membranes from cerebellum and at least four proteins with apparent molecular weights of 51 kDa (P_{51}), 53 kDa (P_{53}), 55 kDa (P_{55}), and 59 kDa (P_{59}) in membranes from hippocampus and other brain regions (Sieghart and Karobath, 1980; Sieghart, 1985; Bureau and Olsen, 1993). Similar heterogeneity was observed in a large variety of different vertebrate species (Hebebrand et al., 1987).

All the proteins irreversibly labeled by [^{3}H]flunitrazepam were associated with central benzodiazepine receptors, because photolabeling by [^{3}H]flunitrazepam was completely blocked by diazepam or clonazepam (Sieghart and Karobath, 1980). All of these proteins were associated with GABA$_A$ receptors, because irreversible binding of [^{3}H]flunitrazepam was stimulated in the presence of GABA, and this stimulation was inhibited in the presence of bicuculline (Sieghart and Karobath, 1980). In addition, binding of [^{3}H]flunitrazepam to the individual proteins was differentially stimulated by pentobarbital and alphaxalone (Bureau and Olsen, 1993). Furthermore, some BZ_1 receptor-selective ligands (see sections II.O. and III.B.4. of this article), such as the triazolopyridazine Cl 218872, the β-carboline βCCE, or the benzodiazepine 2-oxoquazepam (SCH 15725), pref-

erentially inhibited binding of [^{3}H]flunitrazepam to protein P_{51} (Sieghart and Karobath, 1980; Sieghart et al., 1983; Bureau and Olsen, 1993). These data and the predominance of P_{51} in the cerebellum, where BZ_1 receptors are enriched, indicated that protein P_{51} is associated with $GABA_A$ receptors that contain BZ_1 binding sites. The differential potency of Cl 218872, βCCE, or 2-oxoquazepam for inhibition of [^{3}H]flunitrazepam binding to proteins P_{53}, P_{55}, or P_{59} (Sieghart and Karobath, 1980; Sieghart et al., 1983) and their different regional distribution (Sieghart and Karobath, 1980; Sieghart and Drexler, 1983), postnatal development (Eichinger and Sieghart, 1986), and differential protection against treatment of membranes with trypsin (Eichinger and Sieghart, 1985; Schmitz et al., 1989) indicated that each of these proteins is associated with a separate and distinct $GABA_A$ receptor-associated benzodiazepine binding site. Other experiments indicated that the difference in apparent molecular weight of these proteins persists after complete deglycosylation (Sieghart and Fuchs, 1988; Schmitz et al., 1988) and that proteins P_{51} and P_{55} have a different molecular structure (Sieghart, 1988).

A connection between the molecular biological and photolabeling studies on $GABA_A$ receptors was established by immunological investigations. In order to identify the various $GABA_A$ receptor subunits, polyclonal antibodies were raised against peptides having amino acid sequences specific for the individual subunits. These antibodies selectively recognized their immunizing peptide and $GABA_A$ receptors that had been affinity-purified from rat or bovine brain (Stephenson et al., 1989; Fuchs et al., 1990; McKernan et al., 1991; Killisch et al., l991; Endo and Olsen, 1993; Mertens et al., 1993). In addition, each of these antibodies selectively recognized apparently single proteins in purified $GABA_A$ receptor preparations using Western blot techniques. Thus, antibodies directed against the α_1-, α_2, or α_3-subunits of the $GABA_A$ receptor recognized proteins with apparent molecular weights of 51 kDa (P_{51}), 53 kDa (P_{53}), and 59 kDa (P_{59}), respectively, all of which could be photolabeled by [^{3}H]flunitrazepam and, in addition, were identified by the $GABA_A$ receptor-specific monoclonal antibody bd-28 (Fuchs et al., 1990; Buchstaller et al., l991b; Zezula et al., 1991).

These data strongly suggest that the proteins P_{51}, P_{53}, or P_{59} photolabeled by [^{3}H]flunitrazepam, are the α_1-, α_2-, or α_3-subunits of the $GABA_A$ receptor, respectively. Similarly, other studies have been performed that identify the α_4- (Kern and Sieghart, 1994) or α_5 (McKernan et al., 1991; Killisch et al., 1991; Endo and Olsen, 1993; Mertens et al., 1993; Sieghart et al., 1993) subunits as proteins with apparent molecular weights of 67 kDa or 55 kDa, respectively.

Several years ago, it was demonstrated that the partial inverse benzodiazepine receptor agonist [^{3}H]Ro15–4513 (fig. 1) can be used as a photoaffinity label for the investigation of $GABA_A$ receptors (Sieghart et al., 1987). [^{3}H]Ro15–4513 specifically and irreversibly bound not only to the proteins previously identified by photoaffinity labeling with [^{3}H]flunitrazepam but also bound to a protein with an apparent molecular weight of 57 kDa (P_{57}); the latter protein could not be labeled by [^{3}H]flunitrazepam and was almost exclusively present in cerebellar granule cells (Sieghart et al., 1987). Binding of Ro15–4513 to this protein was inhibited by the benzodiazepine receptor antagonist Ro15–1788 and by several other compounds that bind to classical benzodiazepine receptors, but binding was not inhibited by diazepam or the BZ_1 receptor-selective ligands Cl 218872 or βCCE.

In other studies, it was demonstrated that [^{3}H]Ro15–4513 binding to diazepam-insensitive sites in cerebellar granule cells was stimulated by GABA, and this stimulation was partially inhibited by bicuculline (Malminiemi and Korpi, 1989). Furthermore, the antagonism by the inverse benzodiazepine receptor agonist DMCM of [^{3}H]Ro15–4513 binding to diazepam-insensitive sites was modulated in the presence of GABA (Turner et al., 1991). These results seemed to indicate that these diazepam-insensitive sites are associated with a $GABA_A$ receptor. The pharmacological properties of irreversible binding of Ro15–4513 to protein P_{57}, and the exclusive localization of this protein in cerebellar granule cells, which is similar to that of the α_6-subunit (Wisden et al., 1992; Laurie et al., 1992a; Persohn et al., 1992), indicated that protein P_{57} might be associated with α_6-subunit containing $GABA_A$ receptors (Lüddens et al., 1990). This conclusion was confirmed by antibodies selectively recognizing the α_6-subunit, which were able to identify protein P_{57}, photolabeled by [^{3}H]Ro15–4513 (Lüddens et al., 1990; Kern and Sieghart, unpublished results).

Other studies have indicated that muscimol can be used as a photoaffinity label for $GABA_A$ receptors (Cavalla and Neff, 1985; Casalotti et al., 1986; Fuchs and Sieghart, 1989; Bureau and Olsen, 1990). Because of the low specific radioactivity of the [^{3}H]muscimol available and because of a low irreversible labeling efficiency of this compound, photolabeling of $GABA_A$ receptors by [^{3}H]muscimol can only be conveniently investigated in affinity purified $GABA_A$ receptors. [^{3}H]Muscimol irreversibly labeled several different proteins in the apparent molecular weight range between 50 and 58 kDa. The same proteins could be identified by the monoclonal antibody bd-17 (Fuchs and Sieghart, 1989), which selectively identifies the amino acids 1 to 3 of the β_2- and β_3-subunits (Ewert et al., 1990) and by polyclonal antibodies directed against β_2- or β_3-subunits, respectively (Buchstaller et al., l991a). Thus, the proteins photolabeled by [^{3}H]muscimol, in contrast to the α-subunits photolabeled by [^{3}H]flunitrazepam, seem to be β-subunits of the $GABA_A$ receptors. Recently, however, evidence has been presented indicating that α-subunits too could be a substrate for photolabeling by [^{3}H]muscimol (Smith and Olsen, 1994). This is consistent with the observation that GABA-binding sites are present on ho-

mo-oligomeric GABA$_A$ receptors and that the simultaneous presence of α- and β-subunits in the same receptor seems to be necessary to produce high affinity [^{3}H]muscimol binding sites (table 1).

Interestingly, irreversible binding of [^{3}H]muscimol to the individual proteins could be differentially inhibited by the GABA$_A$ receptor agonists THIP or taurine and differentially stimulated by pentobarbital (Bureau and Olsen, 1990, 1991, 1993), indicating that these proteins are associated with pharmacologically different GABA$_A$ receptor subtypes and that these subtypes exhibit different binding properties at the GABA binding site.

In addition to the β-subunit-specific antibodies mentioned, other specific antibodies have recently been developed against β-subunits of GABA$_A$ receptors (Killisch et al., 1991; Pollard et al., 1991; Endo and Olsen, 1992; Rosier et al., 1993) and have been demonstrated to recognize proteins in the molecular weight range of 50 to 58 kDa. Antibodies against the γ_1- (Mossier et al., 1994), γ_2- (Stephenson et al., 1990; Benke et al., 1991b; Killisch et al., 1991; Khan et al., 1993, 1994b), or γ_3- (Tögel et al., 1994) subunit of GABA$_A$ receptors are able to identify protein bands between 45 to 51 kDa, 43 to 49 kDa, or 43 to 46 kDa on Western blots, respectively. The apparent molecular size of these proteins observed on sodium dodecyl sufate-polyacrylamide gel electrophoresis was thus smaller than the cDNA-derived size of the unglycosylated γ_1-, γ_2-, or γ_3-subunit. Such a discrepancy has been observed previously, e.g., for the α-subunit of the nicotinic acetylcholine receptors (Hucho, 1986).

Antibodies directed against the δ-subunit of GABA$_A$-receptors specifically recognized a 52 to 54 kDa protein in purified GABA$_A$ receptor preparations (Benke et al., 1991a; Killisch et al., 1991). No results are available on antibodies specifically directed against β_1-, β_4-, γ_4-, ρ_1-, and ρ_2-subunits of GABA$_A$ receptors. However, the existence of several α- and β-subunit isoforms, which differ in their apparent molecular weight, has been demonstrated (Buchstaller et al., 1991a, b; McKernan et al., 1991; Pollard et al., 1991), and the molecular weight difference is not caused by a differential glycosylation of the same subunit.

C. Immunohistochemical Distribution of γ-Aminobutyric Acid$_A$ Receptor Subunits in the Brain

Only a few antibodies raised against GABA$_A$ receptor subunits have been used so far for immunohistochemical studies. In most of these studies, only one or two antibodies, mostly directed against the α_1- and/or $\beta_{2/3}$-subunits of GABA$_A$ receptors, were used. Thus, the monoclonal antibodies bd-17 (specific for $\beta_{2/3}$-subunits) and bd-24, which specifically recognizes α_1-subunits in human, bovine, cat and monkey but not in rat brain tissue (Ewert et al., 1990), were used for regional and subcellular distribution studies in various species (Richards et al., 1987; Houser et al., 1988; Somogyi et al., 1989; Waldvogel et al., 1990; Nicholson et al., 1992). Similarly, the monoclonal antibody 62–3G1 (De Blas et al., 1988; Juiz et al., 1989; Hendry et al., 1990; Bentivoglio et al., 1991), which recognizes the same epitope on β_2- and β_3-subunits as the monoclonal antibody bd-17 (Ewert et al., 1992) as well as polyclonal antibodies directed against epitopes present on two (Rosier et al., 1993) or three different β-subunits (Gu et al., 1992a), were used in several immunohistochemical studies.

Recently, however, polyclonal antibodies directed against the α_1-, α_2-, α_3-, α_5-, α_6-, γ_2-, and δ-subunits have been used to investigate the regional distribution of the respective subunits (Benke et al., 1991a; Zimprich et al., 1991; Fritschy et al., 1992; Thompson et al., 1992; Gao et al., 1993). These studies indicated a distinct and different regional distribution of these subunits in the brain, a finding which more or less agreed with the respective in situ hybridization data (see section IV.A. of this article). One of the major differences noted is with the γ_2-subunit, which has been detected by immunocytochemistry at high levels in the islands of Calleja and the substantia nigra (Benke et al., 1991b). In situ hybridization revealed, however, that these regions contained γ_2-subunit mRNA at levels lower than would be predicted from the corresponding polypeptide level (Shivers et al. 1989; Wisden et al., 1992). In the hippocampus, the opposite situation was apparent with high levels of the γ_2-subunit mRNA (Shivers et al., 1989; Wisden et al., 1992) but only moderate levels of the polypeptide (Benke et al., 1991b). These results may be caused by differences in the intracellular localization of mRNA and protein. Alternatively, some neurons may display differing gene transcription, mRNA turnover, mRNA translation, and/or protein turnover rates for the same gene or gene product (Williamson and Pritchett, 1994).

In addition, data obtained with subunit specific antibodies, in agreement with pharmacological (Lippa et al., 1981), photolabeling (Eichinger and Sieghart, 1986) and in situ hybridization data (Laurie et al., 1992b; Poulter et al., 1992, 1993; Ma et al., 1993), indicated a distinct postnatal development of the individual subunits, suggesting a switch in the expression of individual GABA$_A$ receptor subtypes (Killisch et al., 1991; Fritschy et al., 1994; Müller et al., 1994; Mathews et al., 1994).

In a first attempt to identify receptor subtypes in situ, the regional and cellular distribution of α_1-, α_3-, $\beta_{2/3}$-, and γ_2-subunits was investigated by double and triple immunofluorescence staining using mono- and polyclonal antibodies specific for these subunits (Fritschy et al., 1992). At both cellular and subcellular levels, five distinct patterns of subunit colocalization were identified: $\alpha_1\beta_{2/3}\gamma_2$; $\alpha_3\beta_{2/3}\gamma_2$; $\alpha_1\alpha_3\beta_{2/3}\gamma_2$; $\alpha_3\gamma_2$; and $\alpha_1\alpha_3\gamma_2$. Because the distribution of other subunits, such as β_1- or δ-subunits, was not investigated in this study, this pattern of subunit colocalization does not necessarily indicate that the identified subunit combinations represent the complete composition of receptors present on the cells investigated. Especially, the $\alpha_3\gamma_2$ or $\alpha_1\alpha_3\gamma_2$ combi-

nations might represent incomplete receptor compositions. In any case, by using confocal laser microscopy, these subunit combinations displayed the same local variations of staining intensity along plasma membranes. Although the covisualized subunits seem therefore to be co-assembled in receptor subtypes, it has to be stressed that the resolution of the technique used in this study does not allow the analysis of the composition of a single receptor.

In a recent study, however, quantitative electron microscopic double immunolabeling analysis on cultured rat cerebellar granule cells was used in combination with the label fracture technique (Caruncho and Costa, 1994). From these studies, it was concluded that the α_1-subunit is preferentially colocalized with the $\beta_{2/3}$- and γ_2-subunits, whereas the α_6-subunit is preferentially colocalized with the $\beta_{2/3}$- and either the δ-, or the γ_2-subunit.

The data, thus, support in situ hybridization experiments, indicating the co-expression of a variety of different $GABA_A$ receptor subunits in single cells (see section IV.A. of this article) and at least indicate a clustering of these subunits in the same area of neurons. In addition, these studies seem to indicate that most neurons express only a single major receptor subtype, with no apparent distinction between synaptic and extrasynaptic sites. In some neurons, however, most notably in Purkinje cells, the subunit composition varied between soma and dendrites, pointing to the existence of receptor heterogeneity within single neurons (Fritschy et al., 1992).

In addition, these studies indicated that specific α-subunit immunoreactivity is associated with certain cell types. Thus, the vast majority of serotonergic, but also dopaminergic and noradrenergic, neurons strongly express α_3-subunit immunoreactivity but are devoid of α_1-subunit staining. In contrast, both α_1- and α_3-subunit immunoreactivities are present in glutamate decarboxylase-positive GABAergic neurons (Fritschy et al., 1992; Gao et al., 1993). The occurrence of neuron-specific $GABA_A$ receptor subtypes may open new possibilities for the targeting of drugs with selective therapeutic actions. It has to be stressed however, that just as the presence of a particular mRNA species does not prove the presence of the corresponding polypeptide, the presence of a specific set of polypeptides does not necessarily mean that they have assembled into a functional receptor complex.

D. Isolation and Composition of γ-Aminobutyric Acid$_A$ Receptor Subtypes from Brain Tissue

In other studies, antibodies that selectively recognize α_1- or α_3-subunits were used to isolate $GABA_A$ receptors from rat brain membrane extracts by immunoaffinity chromatography (Zezula and Sieghart, 1991). The receptors eluted from the anti-α_1- or anti-α_3 affinity column contained predominantly the α_1- or α_3-subunit of $GABA_A$ receptors, respectively. These results seem to indicate that most of the $GABA_A$ receptors contain only a single type of α-subunit. Because even extensive washing of the immunoaffinity column, before elution of the $GABA_A$ receptors, could not remove the small amounts of other α-subunits additionally present in the column eluates, a minor part of the receptors seems to exist that contains two or more different α-subunits (Zezula and Sieghart, 1991). This conclusion was strengthened by other studies, indicating that whenever $GABA_A$ receptors were immunoprecipitated or isolated by immunoaffinity chromatography using α-subunit-specific antibodies, together with the main α-subunit, a small proportion of additional α-subunits were co-isolated. Thus, so far, the coprecipitation of $\alpha_1\alpha_2$-, $\alpha_1\alpha_3$-, $\alpha_2\alpha_3$-, $\alpha_3\alpha_5$-, $\alpha_1\alpha_5$-, and $\alpha_1\alpha_6$-subunits have been demonstrated (Lüddens et al., 1991; Duggan et al., 1991; Mertens et al., 1993; Pollard et al., 1993). Taken together, these results imply the presence of at least two α-subunit copies per receptor oligomer. In most of the cases, identical α-subunits seem to assemble. With much lower frequency, however, two different α-subunits seem to combine with other subunits to form intact $GABA_A$ receptors.

The subunit composition of receptors eluted from the anti-α_1 or anti-α_3-subunit-specific columns was not investigated in great detail because of the lack of availability of specific antibodies against other $GABA_A$ receptor subunits when these studies were performed. In addition to the predominant and the minor α-subunits, however, the presence of $\beta_{2/3}$-, γ_2-, and δ-subunits was demonstrated in these eluates (Zezula and Sieghart, 1991; Mertens et al., 1993). Furthermore, it was shown that receptors eluted from the anti-α_1- or anti-α_3- affinity columns still were able to bind GABA or muscimol and exhibited pharmacological properties of the BZ_1 or BZ_2 benzodiazepine receptors, respectively (Zezula and Sieghart, 1991; McKernan et al., 1991; Mertens et al., 1993). These data are in agreement with studies performed on recombinant $\alpha_1\beta_x\gamma_2$- or $\alpha_3\beta_x\gamma_2$-receptors (table 2) (and section III. B.4. of this article). Thus, $GABA_A$ receptors exhibiting BZ_1 or BZ_2 binding properties could be isolated from the brain and contained a set of subunits similar to that of pharmacologically identical recombinant receptors.

When receptors containing α_5-subunits were immunoprecipitated or purified by anti-α_5-immunoaffinity columns, a striking heterogeneity in their affinity for zolpidem was found, depending on the tissue used to isolate $GABA_A$ receptors (McKernan et al., 1991; Mertens et al., 1993). Receptors immunoprecipitated from striatum and thalamus/hypothalamus exhibited a high affinity for zolpidem (19 nM), whereas receptors isolated from hippocampus exhibited a low affinity (1.2 μM) for this compound. Receptors isolated from cortex or spinal cord exhibited an intermediate affinity for zolpidem (270 nM or 537 nM, respectively) (Mertens et al., 1993). Thus, the

receptor population containing the α_5-subunit seems to be pharmacologically rather heterogeneous. As shown in table 6, recombinant receptors expressing the α_5-subunit in the subunit combination $\alpha_5\beta_x\gamma_2$ lacked affinity for zolpidem (Ki > 10 μM), as did receptors with a subunit combination $\alpha_5\beta_3\gamma_3$ (Lüddens et al., 1994). Similarly, GABA-stimulated chloride flux was only marginally modulated by zolpidem (10 μM) in recombinant $\alpha_5\beta_1\gamma_2$ or $\alpha_5\beta_1\gamma_1$ receptors (Puia et al., 1991). Thus, these subunit combinations seem not to be present to a significant extent in the receptors immunopurified by the anti-α_5-antibodies from striatum, thalamus/hypothalamus, and cortex but might possibly be present in hippocampus and spinal cord, where micromolar affinities for zolpidem were found in the α_5-receptor population. Because the subunit analysis of the receptor population isolated by anti-α_5-antibodies indicated that α_1- or α_3-subunits were co-isolated (Mertens et al., 1993), in brain regions displaying high or intermediate affinities for zolpidem, the α_5-subunit may be associated with a second α-subunit, such as α_1- or α_3-, in the same receptor. Alternatively, a combination of the α_5- with the δ-subunit, and/or the absence in these receptors of β-subunits might have caused these different affinities for zolpidem.

Several recent studies investigated the composition of α_6-subunit containing receptors in cerebellum (Lüddens et al., 1991; Pollard et al., 1993; Quirk et al., 1994a; Khan et al., 1994a, b). By combining information from quantitative immunoprecipitation experiments and Western blot analysis, Quirk et al., 1994a derived a model describing the composition of all GABA$_A$ receptors in the cerebellum. According to this model, 36% of cerebellar GABA$_A$ receptors contained $\alpha_6\gamma_2$- and 23% contained $\alpha_6\delta$-subunits. A combination of α_1- and α_6-subunits in the same receptor was not identified by these authors. The subunit composition of the remaining cerebellar receptors was estimated to be $\alpha_1\gamma_2$ (28%), $\alpha_2\gamma_1$ (8%), and $\alpha_3\gamma_2$ (5%, Quirk et al., 1994a).

The low abundance of receptors containing $\alpha_1\gamma_2$-subunits, and the high abundance of receptors containing the α_6-subunits, is surprising in this study. Because α_6-subunits confer diazepam-insensitivity to [^{3}H]Ro15–4513 binding (Sieghart et al., 1987; Lüddens et al., 1990), these results would imply the presence of more diazepam-insensitive than diazepam-sensitive [^{3}H]Ro15–4513 binding sites in cerebellum. Radioligand binding studies (Sieghart et al., 1987; Billard et al., 1988; Turner et al., 1991), however, indicated that 70 to 80% of GABA$_A$ receptors in cerebellum exhibited diazepam-sensitive [^{3}H]Ro15–4513 binding. These results are indirectly supported by photolabeling (Sieghart and Drexler, 1983; Sieghart et al., 1987) and in situ hybridization (Wisden et al., 1992; Laurie et al., 1992a; Persohn et al., 1992) studies that seemed to indicate that a high percentage of GABA$_A$ receptors in cerebellum contain the α_1-subunit.

In another study (Khan et al., 1994a), it was estimated that 42% of the cerebellar receptors seem to contain $\alpha_1\beta_{2/3}\gamma_2$ subunits coexisting in the same receptor. In addition, it was concluded that a large part of receptors containing α_6-subunits also contain α_1-subunits (Khan et al., 1994a). The latter finding is in agreement with other observations (Lüddens et al., 1991; Pollard et al., 1993) that suggest a partial coexistence of α_6- with α_1-subunits in the same receptor. Neither the complete composition nor the pharmacology of GABA$_A$ receptors containing α_1- and α_6-subunits presently is known. However, if these receptors exhibit diazepam-sensitive [^{3}H]Ro15–4513 binding, the data of Khan et al., 1994a could be more or less consistent with photolabeling (Sieghart and Drexler, 1983; Sieghart et al., 1987) or radioligand binding studies (Sieghart et al., 1987; Billard et al., 1988; Turner et al., 1991). It has to be stressed, however, that a quantitative estimation of receptor subunit composition by immmunoprecipitation studies is subject to a variety of possible artifacts. Thus, the possible crossreactivity of antibodies with each of the GABA$_A$ receptor subunits present in the tissue investigated, has to be carefully checked in immunoprecipitation as well as in Western blot studies using recombinant GABA$_A$ receptors containing the subunits in question. In addition, coprecipitation of unrelated GABA$_A$ receptors caused by aggregation of receptors must be excluded and the efficiency for precipitation of the GABA$_A$ receptors must be determined for each antibody used.

The monoclonal antibody bd-17, which specifically recognizes β_2- and β_3-subunits of GABA$_A$ receptors (Ewert et al., 1990), as well as a β_3-specific polyclonal antibody (Pollard et al., 1991), have been used to investigate which of the other subunits combine with β_2- or β_3-subunits. Results indicated that these β-subunits can be associated with α_1-, α_6-, γ_1-, γ_3-, and the δ-subunits (Pollard et al., 1991; Mertens et al., 1993; Kern and Sieghart, 1994; Tögel et al., 1994). No experiments have been published so far that could answer the question of whether more than one β-subunit coexists in a single GABA$_A$ receptor.

Antibodies specifically recognizing γ_1-subunits, on immunochromatography not only retained γ_1-, but also α_1-, α_2-, α_3-, α_5-, and $\beta_{2/3}$-subunits of GABA$_A$ receptors (Mossier et al., 1994). Antibodies directed against γ_2-subunits were able to coprecipitate several different α-subunits, as well as $\beta_{2/3}$ and δ-subunits (Duggan et al., 1991; Mertens et al., 1993). Similarly, antibodies directed against γ_3-subunits were able to precipitate α_1-, α_2-, α_3-, α_4-, and $\beta_{2/3}$-subunits from brain membrane extracts prepared from rat cerebral cortex (Tögel et al., 1994). In addition, α_6-subunits were co-isolated by these antibodies from cerebellar extracts. Interestingly, however, anti-γ_1-antibodies were not able to isolate γ_2- or γ_3-subunits. Anti-γ_2-antibodies did not isolate γ_1- or γ_3-, and anti-γ_3-antibodies did not isolate γ_1- or γ_2-subunits.

These experiments seemed to indicate that, in most $GABA_A$ receptors, only a single type of γ-subunit is present. In other studies, however, quantitative immunoprecipitation with anti-γ_{2S} and anti-γ_{2L}-antibodies (Khan et al., 1994a, b) indicated a significant colocalization of these two forms of the γ_2-subunit in the same receptor. In addition, quantitative immunoprecipitation studies using antibodies specific for the γ_1-, γ_2-, and γ_3-subunit of the $GABA_A$ receptor indicated that γ_2- and γ_3-subunits might coexist in the same receptor but that γ_1-subunits do not exist in combination with another γ-subunit (Quirk et al., 1994b). Differences in receptor composition in different brain tissues and in the individual antibody sensitivity and/or crossreactivity with other $GABA_A$ receptor subunits might account for these discrepancies.

Only two studies (Benke et al., 1991a; Mertens et al., 1993) have been performed investigating the presence of δ-subunits in isolated $GABA_A$ receptors, and a coprecipitation of α_1- and α_3-subunits with δ-subunits has been demonstrated (Mertens et al., 1993). In addition, in a recent immunolabeling study on cultured rat cerebellar granule cells, an association of the δ- with the α_6-subunit has been shown (Caruncho and Costa, 1994). Furthermore, the presence of $\beta_{2/3}$- and γ_2-subunits has been demonstrated in receptors eluted from a δ-subunit-specific immunoaffinity column. These results are supported by in situ hybridization histochemistry demonstrating the occurrence of δ-, α_1-, α_3-, and γ_2-subunits in hippocampus, olfactory bulb, and cerebral cortex and of α_6- and δ-subunits in cerebellar granule cells (Wisden et al., 1992; Persohn et al., 1992). In addition, an association of the δ-subunit with the γ_2-subunit is in line with immunohistochemical observations of the δ-subunit being co-expressed with the γ_2-subunit in many cells (Benke et al., 1991a; Fritschy et al., 1992).

Based on its role in receptors containing α- and β-subunit variants (Pritchett et al., 1989), the γ_2-subunit also would be expected to convey benzodiazepine sensitivity to receptors containing the δ-subunit. This actually has been found in receptors immunoprecipitated with δ-subunit-specific antibodies. These receptors displayed a high affinity site for the $GABA_A$ agonist muscimol and for the benzodiazepine receptor antagonist Ro15–1788.

The pharmacological profile of the receptor population is unique and different from that of receptor populations immunoprecipitated by either α_1-, α_3-, or α_5-subunit antiserum (Mertens et al., 1993). As judged by its subunit composition, the δ-subunit-containing immunopurified receptor population is presumably still heterogeneous, and thus, the binding properties of these receptors reflect the average properties of several different receptors. The possible existence in the brain of receptors consisting of α-, β-, and δ-subunits, a subunit combination that has been demonstrated to result in recombinant receptors devoid of benzodiazepine binding sites (Shivers et al., 1989), thus cannot be excluded.

E. Theoretical Considerations on the Subunit Stoichiometry and Arrangement of γ-Aminobutyric Acid$_A$ Receptors

Thus, results from immunopurification studies support the evidence from recombinant receptor studies that α-, β, and γ-subunits co-assemble to form native $GABA_A$ receptors. Most of the α-subunits seem to be able to assemble with the β_2- or β_3-subunits as well as with the γ_1-, γ_2-, or γ_3-subunits, resulting in a multiplicity of $GABA_A$ receptors. Although two α-subunits seem to be present in native $GABA_A$ receptors, no information is available on the possible presence of more than one β-subunit in these receptors. In addition, results on the number or γ-subunits in $GABA_A$ receptors are conflicting. The presence of three α-, β-, or γ-subunits in the same $GABA_A$ receptor, however, was excluded by a recent electrophysiological investigation, analyzing the effect of certain point mutations of $GABA_A$ receptor subunits on the outward rectification of GABA-evoked current in recombinant receptors (Backus et al., 1993). This study suggested that $GABA_A$ receptors could have subunit stoichiometries of $2\alpha + 1\beta + 2\gamma$, $2\alpha + 2\beta + 1\gamma$, or $1\alpha + 2\beta + 2\gamma$, of which the subunit composition $2\alpha + 1\beta + 2\gamma$ may be favored (Backus et al., 1993).

However, if it is assumed that two α-subunits are present in native $GABA_A$ receptors, as suggested by the immunopurification or immunoprecipitation data discussed above, $GABA_A$ receptors containing only one α-subunit can be excluded. By using a subunit-association rank order ($\alpha\beta > \alpha\gamma > \beta\gamma > \alpha\alpha \simeq \beta\beta \simeq \gamma\gamma$) that can be derived from the efficiency of formation of recombinant receptors composed of two different subunits (see section III.B.3. of this article), the $GABA_A$ receptor structure shown in figure 5A can be logically derived. This structure consists of 2α-, 2β- and 1γ-subunit and can arise, for instance, by the assembly of two energetically favorable αβ dimers (Angelotti et al., 1993a) with a γ-subunit. The subunit arrangement in figure 5A assumes that by combining the two dimers again, the same association-efficiency rank order can be applied. Thus, because an αα or a ββ association, is energetically less favored than that of an αβ association the α-subunit of an αβ dimer pref-

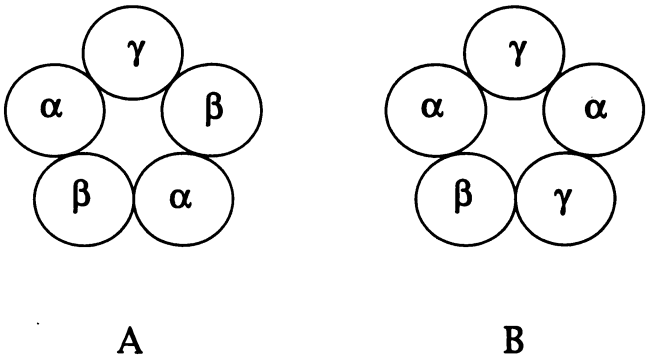


FIG. 5. Theoretically derived $GABA_A$ receptor structures.

erentially will associate with the β-subunit of the other αβ dimer. The pentameric structure is then completed by the co-assembly with a γ-subunit, which adds to the stability of this structure by forming two relatively stable (αγ plus βγ) interactions. A possible co-assembly with an α- or a β-subunit probably is less favorable because of the necessity of forming an energetically instable αα or ββ association, respectively. Applying the same association-efficiency rank order, an identical structure can be obtained when αβ and αγ dimers combine with single β-subunits. The subunit arrangement is determined by the high stability of the αβ (compared with the αγ or αα) interaction, and the structure is completed by a β- and not by an α- or a γ-subunit, because the α- or γ-subunits again would have to form energetically unstable αα or γγ associations, respectively. Because of the low stability of a βγ dimer, the third possibility for the formation of the structure shown in figure 5A, an assembly of an αβ and a βγ dimer with an α-subunit, seems to have no relevance in vivo.

The GABA$_A$ receptor structure containing 2α-, 1β-, and 2γ-subunits (fig. 5B), which seemed to be slightly favored in the electrophysiological investigations of Backus et al.,1993, seems to be energetically less favorable than the structure shown in figure 5A, when the same association-efficiency rank order is applied. This is easily realized when the number and types of subunit-interactions in these structures are summarized. Whereas the structure in figure 5A involves three αβ, one αγ, and one βγ interactions, the structure in figure 5B is formed by only one αβ but three αγ and one βγ interactions. Even under conditions in which only a few β-subunits are available and in which two αγ dimers could possibly combine with one β-subunit in order to form the structure shown in figure 5B, the β-subunit probably would associate with an α-subunit first, to form a stable αβ dimer. On assembly of an αβ with an αγ dimer, however, the structure shown in figure 5A will be favored (see paragraph above). The third pathway for the assembly of the structure shown in figure 5B, a co-assembly of an α-subunit with an αγ and a βγ dimer, seems to be energetically even less likely. Thus, the structure shown in figure 5B can only be formed under conditions in which the association of α- and γ-subunits is energetically comparable to or superior to that of α- and β-subunits. Such conditions actually seem to exist (see sections III.B.3 and III.B.7 of this article), because, depending on the cell system and experimental conditions used, the existence of recombinant receptors consisting of αγ subunits could (Draguhn et al., 1990; Verdoorn et al., 1990; Sigel et al., 1990) or could not be (Angelotti et al., 1993a) demonstrated. No information is presently available on the stability of subunit dimers in the brain. Depending on whether the αβ or the αγ association is more stable, the structure in figure 5A or 5B will be favored. These considerations, however, don't take into account that the assembly of two subunits could cause a change in the efficieny of association with additional subunits. If this is the case, other subunit stoichiometries and arrangements are possible.

The subunit structures shown in figure 5A or 5B could accomodate all the pharmacological information available on recombinant and native GABA$_A$ receptors. Because GABA, picrotoxin, and pentobarbital binding sites seem to be able to form on all homo-oligomeric GABA$_A$ receptors (see section III.B.2. of this article), up to five binding sites for these compounds could be present on each of the receptor structures shown in figure 5. This is in agreement with previous evidence that indicates the existence of several binding sites for GABA and pentobarbital on a single GABA$_A$ receptor (see sections II.A. and II.N. of this article).

In contrast, benzodiazepine binding sites seem only to be formed in the presence of a γ-subunit and seem to be localized at the interface of the αγ or possibly βγ subunit (table 1). This is supported by photolabeling studies indicating that predominantly the α-subunits are photolabeled by [^{3}H]flunitrazepam (see section IV.B. of this article). Thus, the structures in figure 5A or 5B possibly could form two or four benzodiazepine binding sites, respectively, allowing for the presence of several benzodiazepine binding sites in a single GABA$_A$ receptor (see section II.B. and II.N. of this article). Alternatively, the benzodiazepine binding sites might be formed by a conformational change in the α-subunits induced on assembly with a γ-subunit. In this case again, several binding sites might be present on each of the structures shown in figure 5A and 5B.

V. Plasticity of γ-Aminobutyric Acid$_A$ Receptors

Neurotransmitter receptors are subject to regulation after their activation by agonists. The initial event in the regulatory pathway is a rapid desensitization of receptors, which can be complete within seconds and produces a loss of immediate responsiveness. If agonist exposure persists for minutes to hours, receptors are removed from the cell surface membrane and enter an internal membrane pool, a process referred to as internalization or sequestration. The internalized receptors can either be recycled to the surface or degraded. The latter event, favored by exposure to agonists for hours to days, is often termed down-regulation and is accompanied with a reduction of, or change in, receptor gene expression (Klein et al., 1989). In contrast, discontinuation of agonist treatment (Miller et al., 1988b), or treatment of receptors with antagonists or inverse agonists (Miller et al., 1990a; Primus and Gallager, 1992; Lewin et al., 1994) induces receptor up-regulation, which might occur by mechanisms opposite to those described above. The mechanisms involved in sensitizing or desensitizing GABA$_A$ receptors, however, have not been investigated extensively. Whereas the agonist-dependent regulation of β-adrenergic receptors and their coupling to GTP-binding proteins are particularly well understood

(Benovic et al., 1988), the elucidation of comparable mechanisms that control the sensitivity, number and composition of $GABA_A$ receptors is in a much earlier stage of development.

A. Agonist-induced Desensitization of γ-Aminobutyric Acid$_A$ Receptors

A variety of evidence indicates that the properties of $GABA_A$ receptors change on short term application of $GABA_A$ agonists. Thus, a continued application of GABA to neurons produces a decrease in the GABA-induced current, because of both a change in the transmembrane chloride gradient (Segal and Barker, 1984; Huguenard and Alger, 1986) and a decrease in the induced conductance, a true receptor desensitization. This latter phenomenon has been observed electrophysiologically in neocortical and hippocampal neurons (Numann and Wong, 1984; Oh and Dichter, 1992), as well as in recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes (Sigel et al., 1990) and HEK cells (Verdoorn et al., 1990).

By varying the time of pre-incubation of sealed brain membrane vesicles with GABA between 10 ms and 50 s with quench flow technique, the desensitization rates could be measured over their whole time course independently of the chloride ion flux rate (Cash and Subbarao, 1987a). These experiments indicated that most of the receptor activity decreased in a fast phase of desensitization, which was complete in 200 ms at saturation with GABA. Remaining activity was desensitized in a few seconds (Cash and Subbarao, 1987a). These two phases of desensitization were each kinetically first order and were shown to correspond with two distinguishable $GABA_A$ receptors on the same membrane. The faster and slower desensitizing receptor exhibited a half-response concentration of 150 μM and 114 μM GABA, respectively. Electrophysiological investigations using the whole cell patch clamp method yielded similar results, but, probably because of the lower time resolution of the GABA application technique used, these investigations were not able to resolve the fast phase of desensitization (Oh and Dichter, 1992). Nevertheless, application of GABA to cultured hippocampal neurons induced a current that peaked within less than 1 s and stayed elevated for only a short time. The current then decreased exponentially with a single time constant. Desensitization of $GABA_A$ receptors was concentration-dependent, because higher concentrations of GABA perfused on the cell resulted in faster and more extensive desensitization (time constant of current decay < 3 s). Recovery from desensitization induced by 10 μM GABA was complete within 3 to 4 min after removal of GABA (Mierlak and Farb, 1988; Oh and Dichter, 1992).

Interestingly, desensitization of GABA-induced currents was markedly voltage-dependent. Thus, desensitization was smaller and slower as the membrane was depolarized, and almost no desensitization was observed at +30 mV (Oh and Dichter, 1992). In contrast, in frog sensory neurons (Akaike et al., 1986) or in retinal ganglion cells from 7- to 11-day-old rats (Tauck et al., 1988), desensitization of GABA-induced current was reported not to be altered by membrane potential. Thus, the properties of the $GABA_A$ receptors might be different in different brain regions, and the differences in desensitization characteristics could have significant consequences for the normal regulation of GABA-induced inhibition. For instance, such voltage dependency might be important in the regulation of interaction between excitatory and inhibitory transmitters in the central nervous system. Neurons depolarized by excitatory neurotransmitters would exhibit less desensitization with GABA; therefore GABA could exert a more profound inhibitory effect on neurons that are excessively excited.

Pentobarbital was able to increase both the GABA-mediated chloride-exchange rate (reflecting channel-opening equilibrium) and receptor desensitization rates in $^{36}Cl^-$ tracer ion flux studies with brain membrane vesicles (Cash and Subbarao, 1988). Similarly, the benzodiazepine binding site agonists chlordiazepoxide and flunitrazepam not only potentiated the peak response of 10 μM GABA induced on neuronal cultures from spinal cords of 6- to 7-day-old chick embryos, but also increased the apparent rate constant and extent of desensitization for GABA response (Mierlak and Farb, 1988). This effect could be blocked by the benzodiazepine binding site antagonist Ro15–1788. The experiments indicated that chlordiazepoxide-stimulated desensitization did not simply reflect increased receptor occupancy by GABA in the presence of this compound. The stimulation of desensitization by pentobarbital and benzodiazepine binding site agonists might indicate that the total amount of Cl^- ions entering the cell triggers desensitization.

Alternatively, a direct stimulation by these compounds of the desensitization mechanism is possible. Thus, quench flow studies indicated that barbiturate binding sites different from those involved in the enhancement of GABA induced chloride flux or in direct opening of chloride channels might be involved in modulation of desensitization of $GABA_A$ receptors (Cash and Subbarao, 1988). In addition, the recent discovery of the dihydroimidazoquinoxaline U-93631 (see section II.M.9. of this article), which accelerated the decay of GABA-induced chloride current without producing noticeable changes in the amplitude of the current (Dillon et al., 1993), and the finding that the picrotoxinin binding site ligand dieldrin seems to block GABA-induced chloride ion flux by accelerating desensitization (Nagata and Narahashi, 1994), support a direct effect of these compounds on desensitization.

B. Agonist-induced Down-regulation of γ-Aminobutyric Acid$_A$ Receptors

There is general agreement that chronic exposure of cultured neurons to GABA results in a reduction in the number of [^{3}H]muscimol, [^{3}H]flunitrazepam, and

[^{35}S]TBPS binding sites, as well as in the extent of allosteric coupling between the remaining GABA and benzodiazepine recognition sites (Hablitz et al., 1989; Roca et al., 1990; Mehta and Ticku, 1992; Calkin and Barnes, 1994). In addition, the GABA-induced $^{36}Cl^-$-influx or the peak current amplitudes induced by GABA were reduced in these cells. Because neither the rate of rapid desensitization nor the affinity of the receptors for flunitrazepam was changed by chronic GABA exposure, these results were consistent with a down-regulation of GABA$_A$ receptors (Hablitz et al., 1989; Mehta and Ticku, 1992).

In some systems, however, prolonged incubation with GABA led to an increase in [^{3}H]muscimol or [^{3}H]GABA binding (Meier et al., 1984), to an increase in GABA$_A$ receptor mRNA expression (Kim et al., 1993) and to an increase in the density of GABA$_A$ receptors (Hansen et al., 1991). Because the period of the greatest increase in the number of receptor sites coincides with the development of the cerebellar granule cells (Kim et al., 1993), this effect seems to be caused by an effect of GABA on the cytodifferentiation of developing neurons.

Down-regulation was dose- and time-dependent. The GABA dose-response curve exhibited an EC_{50} of 94 μM, a $t_{1/2}$ of 25 h, and a maximum decrease in [^{3}H]flunitrazepam binding of 42% (Roca et al., 1990; Mehta and Ticku, 1992). In addition, down-regulation of GABA$_A$ receptors could be inhibited by the GABA$_A$ receptor antagonist R 5135 (Mehta and Ticku, 1992) and was reversible after removal of GABA (Roca et al., 1990).

In another report, it was demonstrated that down-regulation involved not only a reduction in GABA$_A$ receptor function but also a removal of GABA$_A$ receptor subunits from the cell surface that was similar in extent to the down-regulation of GABA$_A$ receptor ligand binding sites (Czajkowski and Farb, 1989; Calkin and Barnes, 1994). In addition, it was demonstrated that the subunits removed from the cell surface seemed to become part of an intracellular pool of benzodiazepine binding sites (Tehrani and Barnes, 1991), which might be connected with clathrin-coated vesicles (Tehrani and Barnes, 1993). GABA$_A$ receptors, which are sequestered from the cell surface during acute GABA treatment, do not continue to accumulate internally during chronic treatment. It was demonstrated that specifically labeled GABA$_A$ receptor polypeptides were removed from the endogenous GABA$_A$ receptor pool with a velocity similar to that of internalization (Czajkowski and Farb, 1989; Calkin and Barnes, 1994). In another study, it was demonstrated that GABA$_A$ receptors are degraded through an energy-dependent nonlysosomal pathway (Borden and Farb, 1988).

C. Agonist-induced Changes in Subunit Gene Expression

An alternative (or concomitant) mechanism of receptor down-regulation might be the regulation of subunit gene expression by GABA$_A$ receptor occupancy. Thus, it has been demonstrated that prolonged (48 h) incubation of primary neuronal cultures with GABA, at concentrations maximally effective in reducing the number of benzodiazepine binding sites, resulted in a marked (40 to 80%) reduction in GABA$_A$ receptor α-subunit mRNAs (Montpied et al., 1991a) as well as of α-subunit polypeptide expression (Mhatre and Ticku, 1994), which could be completely prevented by the GABA$_A$ receptor antagonists SR 95531 or R 5135. This effect could not be reproduced by a change in membrane potential and thus seemed to have been elicited by GABA$_A$ receptor activation. The possible additional down-regulation of GABA$_A$ receptor β- and/or γ-subunit mRNAs and polypeptides during prolonged treatment of receptors with GABA, as well as the molecular mechanisms by which activation of ligand-gated ion channels regulate the cellular level of receptor subunit-encoding mRNAs, must be investigated in future experiments.

D. Regulation of γ-Aminobutyric Acid$_A$ Receptor Function by Phosphorylation

The molecular mechanism of agonist-dependent desensitization is not understood. There is, however, an artificially produced phenomenon that might be related to receptor desensitization and that has been associated with phosphorylation of GABA$_A$ receptors.

Thus, it has been demonstrated that GABA-mediated chloride currents measured in the whole-cell clamp configuration, probably caused by wash-out of endogenous factors, diminish progressively (receptor 'run-down') when these neurons are perfused with a 'minimal' intracellular medium containing only inorganic ions, ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, and buffer (Gyenes et al., 1988; Stelzer et al., 1988; Stelzer, 1992). This effect develops more slowly than receptor desensitization, and whereas GABA$_A$ receptor desensitization is reversible on a time scale of a few minutes, GABA$_A$ receptor run-down is irreversible on the time scale of an average experiment (Gyenes et al., 1994). Addition of Mg^{2+}-ATP—but not of a nonhydrolyzable ATP analog—to these cells retards this receptor run-down, as does ATP-γ-S, an ATP analog that donates a phosphatase-resistant thiophosphate group to phosphoproteins (Stelzer et al., 1988; Gyenes et al., 1988).

These observations indicate that phosphorylation of either the GABA$_A$ receptor itself or of a closely associated protein may be required to maintain receptor function. This is consistent with the observation that receptor run-down is accelerated by alkaline phosphatase or the Ca^{2+}-dependent phosphatase calcineurin and that ATP-γ-S attenuates the effect of these phosphatases on run-down (Stelzer, 1992; Gyenes et al., 1994). The endogenous protein phosphatase and protein kinase involved in regulation of basal GABA$_A$ receptor function presently has not been identified.

In at least some systems, receptor run-down is dependent on agonist application (Stelzer, 1992) and is enhanced by pentobarbital or allopregnanolone (Gyenes et al., 1994). $GABA_A$ receptor run-down, at least in these systems, might thus be a consequence of $GABA_A$ receptor activation and/or desensitization.

Interestingly, however, run-down changed the pharmacology of $GABA_A$ receptors. Thus, the potency of GABA for this receptor was increased, whereas its efficacy was decreased. In addition, the potentiation of GABA-induced ion flux by positive modulators was decreased after receptor run-down (Gyenes et al., 1994). These observations are consistent with a large variety of reports indicating that phosphorylation and dephosphorylation of receptors and/or associated ion channels can serve to control receptor-operated ion channel function (Huganir and Greengard, 1990; Stelzer, 1992).

The intracellular loops of several subunits of the $GABA_A$ receptor contain consensus sequences for phosphorylation by cyclic adenosine monophosphate-dependent protein kinase, protein kinase C, and protein tyrosine kinase. The number and position of the phosphorylation sites vary in the α-, β-, γ-, δ-, and ρ-subunits and in the different subtypes of the same subunit (Macdonald and Olsen, 1994). Purified preparations of the $GABA_A$ receptor as well as recombinant $GABA_A$ receptors could be phosphorylated by cyclic adenosine monophosphate-dependent protein kinase and by protein kinase C (Stelzer, 1992). The functional effects of $GABA_A$ receptor phosphorylation, however, were complex. Thus, physiological studies have suggested that phosphorylation of $GABA_A$ receptors by various kinases inhibits, potentiates, or has no effect on $GABA_A$ receptor function. (Leidenheimer et al., 1991; Stelzer, 1992). These differential effects of phosphorylation may have been caused by different receptor subunit combinations in the various systems but were complicated by the nonspecific effects that many drugs used to activate protein kinases have on $GABA_A$ receptors (Leidenheimer et al., 1991). In addition, effects of protein phosphorylation on $GABA_A$ receptor assembly and degradation might have been involved in some of these effects (Green et al., 1991; Ross et al., 1991).

However, when using heterologous expression of $GABA_A$ receptors composed of α_1-, β_1-, γ_{2S}- or γ_{2L}-subunits coupled with site-directed mutagenesis, it has been demonstrated that both protein kinase A and protein kinase C phosphorylation inhibit GABA-activated responses (Moss et al., 1992; Kellenberger et al., 1992). The degree of negative modulation and the regulation of rapid desensitization have been shown to be dependent on the subunit composition of the expressed $GABA_A$ receptor (Moss et al., 1992; Kellenberger et al., 1992).

Other experiments have indicated that a phorbol ester that activates protein kinase C—but not an analog that does not activate this enzyme—not only inhibited GABA-activated responses of *Xenopus* oocytes expressing $\alpha_1\beta_1\gamma_{2L}$ subunit cDNAs but also enhanced the ability of benzodiazepines or barbiturates to potentiate the effects of GABA (Leidenheimer et al., 1993). These results suggest that protein kinase C-dependent phosphorylation of the $GABA_A$ receptor may alter the coupling between allosteric sites within the receptor complex. Whether this effect of protein kinase C on the efficacy of benzodiazepines or barbiturates is related to the ability of this enzyme to induce ethanol sensitivity to $GABA_A$ receptors (Wafford and Whiting, 1992; Weiner et al., 1994) (see section III.B.4.) must be clarified in future experiments.

In addition to the protein kinases A and C, and possibly the protein tyrosine kinase, other protein kinases seem to be able to modulate $GABA_A$ receptor function. Recently, specific sites of phosphorylation for calcium/calmodulin Type 2-dependent protein kinase and cyclic guanosine monophosphate-dependent protein kinase have been identified within $GABA_A$ receptor subunits (Mcdonald and Moss, 1994). In addition, evidence has been presented for the regulation of $GABA_A$ receptor function by a novel chloride-dependent kinase and a sodium-dependent phosphatase (Lanius et al., 1993).

Thus, a variety of different protein kinases seem to be able to interact with $GABA_A$ receptors. The action of these kinases (and of the corresponding phosphatases, which have been much less investigated) and their effect on $GABA_A$ receptor function, might depend not only on receptor subunit composition but also on the state of phosphorylation of the various receptor subunits. This is indicated by the observation that protein kinase A- and protein kinase C-phosphorylation inhibit, whereas the endogenous kinase, preventing receptor run-down, enhances $GABA_A$ receptor function. Additional phosphorylation at a different subunit or site, or dephosphorylation at specific site(s), might enhance or reduce $GABA_A$ receptor function.

Because the various enzymes possibly involved in phosphorylation and dephosphorylation of $GABA_A$ receptors could be activated by many different transmitter and second-messenger systems, this offers the possibility of short- and long-term modulation of $GABA_A$ receptor function by other transmitter systems. Furthermore, it should be stressed that drugs interfering with these regulatory mechanisms could influence the GABAergic system (Leidenheimer et al., 1993) and could lead to specific GABAergic effects.

E. Development of Tolerance to Allosteric γ-Aminobutyric Acid$_A$ Receptor Ligands

Not only long term exposure of neurons to GABA but also chronic treatment with allosteric modulators of $GABA_A$ receptors causes a change in $GABA_A$ receptor function. A large variety of clinical and experimental studies have indicated that prolonged administration of benzodiazepines (Greenblatt and Shader, 1978; File, 1985; Rosenberg and Chiu, 1985; Marley and Gallager,

1989), but also of barbiturates (Saunders et al., 1992), steroids (Parducz et al., 1993), or ethanol (Morrow et al., 1988; Mhatre et al., 1988) to animals or humans results in the development of functional tolerance to the actions of these drugs. However, the mechanisms involved in the development of tolerance to these drugs in living animals have proved difficult to investigate. Thus, the degree of tolerance developed and the effects observed can depend on a variety of behavioural and environmental factors (File, 1985) and might be the outcome of compensatory interactions of many different transmitter systems in the brain. The compensatory processes can be induced in the target cell of the drug itself (receptor or postreceptor mechanisms) and/or in cells being closely or distantly connected with the target cell (Haefely, 1986). In addition, the initial effect of the drug on GABA$_A$ receptor function might subsequently be overruled by secondary effects of other transmitter systems interacting with the same cell and influencing the function of GABA$_A$ receptors via second-messenger systems. Thus, the results obtained in ex vivo experiments very much depend on the brain region and cells investigated and can change from cell to cell; in order to investigate the molecular mechanisms involved in the development of tolerance, single cell systems must be used.

1. Tolerance to benzodiazepines. A variety of evidence indicates that GABA$_A$ receptors are directly affected by prolonged treatment of animals with benzodiazepines. However, the effects observed were dependent on the particular benzodiazepines used for chronic treatment. Thus, variable effects were observed with different benzodiazepine agonists (Galpern et al., 1990; Lopez et al., 1992). Partial benzodiazepine agonists failed to induce tolerance to both the anticonvulsant effect and the positive modulatory action of these drugs on GABA$_A$ receptor function in mouse brain (Miller et al., 1990b; Ghiani et al., 1994), and antagonists or inverse agonists induced receptor sensitization (Miller et al., 1990a; Primus and Gallager, 1992; Lewin et al., 1994). In addition, the intensity and duration of chronic treatment, the time of analysis post-treatment, and the brain area evaluated (Marley and Gallager, 1989; Tietz et al., 1989; Galpern et al., 1990; Lopez et al., 1992; Li et al., 1993) influenced the effects observed. Thus, chronic benzodiazepine exposure have been reported to cause either no change, an increase, or a decrease in the benzodiazepine number or affinity (for review, see Miller et al., 1988a; Prasad and Reynolds, 1992; Hu and Ticku, 1994a). GABA$_A$ receptor function was also reported to be either unchanged or decreased after chronic benzodiazepine treatment. However, chronic benzodiazepine treatment has also been reported to produce a decreased coupling between the GABA$_A$ receptor and its benzodiazepine binding site (Tietz et al., 1989; Marley and Gallager, 1989; Li et al., 1993; Hu and Ticku, 1994a, b), or a shift in the efficacy of benzodiazepines toward inverse agonist properties (Petersen and Jensen, 1987; Little et al., 1987; Nutt et al., 1992). Other studies, however, could not confirm a shift in efficacy of benzodiazepines (Wilson and Gallager, 1989a, b; Hu and Ticku, 1994b).

Recently, it was demonstrated that tolerance development to benzodiazepines is influenced by the degree of activation of the GABA$_A$ receptor (Prasad and Reynolds, 1992). Thus, the presence of both GABA and flurazepam was necessary to induce long-lasting changes in GABA$_A$ receptor function, and tolerance was reduced or eliminated when the GABA$_A$ receptor antagonist bicuculline was included with the GABA and flurazepam treatment (Prasad and Reynolds, 1992). This observation is consistent with the fact that benzodiazepines are not able to directly activate the GABA$_A$ receptor channel complex in the absence of GABA. The different degree of activation by GABA of GABA$_A$ receptors in different brain regions during chronic treatment with benzodiazepines and the different efficacy of individual benzodiazepines for the enhancement of GABA actions at different GABA$_A$ receptor subtypes might explain at least part of the discrepancies described in the above paragraph.

The molecular basis of uncoupling and decreased efficacy of benzodiazepine potentiation of GABA action, in the course of chronic benzodiazepine treatment, has yet to be established. Recent studies have measured the changes in GABA$_A$ receptor gene expression after chronic benzodiazepine treatment (Heninger et al., 1990; Kang and Miller, 1991; O'Donovan et al., 1992; Zhao et al., 1994). Although again there were some discrepancies in the results obtained, which might have been for the reasons discussed in the above paragraph, the levels of several α-subunit mRNAs were changed differentially in several brain regions. In addition, the levels of the γ_2-subunit mRNA were significantly reduced in cortex and hippocampus (but not in cerebellum) and the values returned to control levels 48 h after termination of the treatment. As discussed in section III.B.4., benzodiazepine action requires the assembly of a γ_2-subunit in combination with the α- and β-variants. In addition, the type of α-subunit is crucial in determining the degree of coupling between GABA and benzodiazepine sites and benzodiazepine potentiation of GABA responses in transfected cells. For instance, the α_3-subunit seems to give maximal efficacy of benzodiazepine agonists in enhancing GABAergic responses (Pritchett et al., 1989; Puia et al., 1991). The regional distribution and time course of reduced γ_2-levels matched the decrease in benzodiazepine binding produced by the same chronic flurazepam treatment (Zhao et al., 1994). Thus, a change in the level of expression of the γ-subunit and/or certain α-subunits might have been responsible for benzodiazepine treatment induced changes in GABA$_A$ receptor coupling in these experiments. In other experiments, however, the rapid time course of uncoupling ($t_{1/2}$ of 30 min of treatment with clonazepam) seems to indicate that this process might have been

initiated by a post-translational change in the $GABA_A$ receptor structure (Klein et al., 1994).

2. Tolerance to Barbiturates. Experimental models for inducing tolerance to and dependence upon barbiturates have been reported (Ho and Harris, 1981). Earlier studies, however, used peripheral administration of barbiturates, which allows pharmacokinetic tolerance to barbiturates to develop. This is because barbiturates are potent inducers of hepatic drug-metabolizing enzymes (Conney, 1967). Recently, an intracerebroventricular application of pentobarbital into rat brain was used to avoid hepatic enzyme induction (Tseng et al., 1993a, b; 1994). It was demonstrated that functional tolerance to pentobarbital can be induced as early as 2 days after the start of the infusion but that the dosing regimen influenced at least some changes in $GABA_A$ receptor binding parameters observed (Tseng et al., 1993a).

Results of [^{3}H]muscimol, [^{3}H]flunitrazepam, or [^{35}S]TBPS binding assays showed marked regional variations and were different in tolerant or withdrawn (24 h after termination of infusion) animals, possibly explaining controversial data obtained in different studies (Tseng et al., 1993a). Considering the GABA binding sites alone, it seems that tolerance to pentobarbital in at least the frontal cortex induces a subsensitive $GABA_A$ receptor (decrease of B_{max}), whereas in a withdrawal situation a shift in the reverse direction seems to occur. The cerebellum and striatum, however, did not exhibit these changes. In addition, differential changes in $GABA_A$ receptor subunit mRNA levels were observed in pentobarbital-tolerant and pentobarbital-withdrawn animal (Tseng et al., 1993b, 1994). Single cell studies, however, have not been performed to investigate the molecular mechanism of development of tolerance to barbiturates.

3. Tolerance to ethanol. Chronic exposure to ethanol results in the development of tolerance and physiological dependence in animals and humans and decreased the efficacy of GABA-induced $^{36}Cl^-$ influx in synaptoneurosomes (Morrow et al., 1988). In addition, chronic ethanol treatment produced an up-regulation of binding sites for the benzodiazepine inverse agonist Ro15–4513 in the cerebral cortex and cerebellum of the rat brain, with no change in the binding of benzodiazepine agonists or antagonists (Mhatre et al., 1988). Again, the mechanism of this chronic ethanol-induced subsensitivity of the $GABA_A$ receptor has not yet been explored. Recent studies, however, indicate that chronic ethanol administration differentially alters the levels of several $GABA_A$ receptor subunit mRNAs (Montpied et al., 1991b; Mhatre and Ticku, 1992) as well as that of $GABA_A$ receptor subunit proteins (Mhatre et al., 1993) in the brain.

F. Brain Activity Dependent Regulation of γ-Aminobutyric Acid$_A$ Receptor Function

$GABA_A$ receptor mRNA expression and function not only is regulated by GABAergic drugs but also by hormones (Go et al., 1988; Parducz et al., 1993), other transmitters (Memo et al., 1991; Harris et al., 1994b) and by physiological or pathological conditions changing the activity of GABAergic transmission. Thus, it has been demonstrated that $GABA_A$ receptor subunit mRNA (Huntsman et al., 1994) and protein (Hendry et al., 1994) expression is regulated by visually driven activity. In other studies, it has been demonstrated that $GABA_A$ receptor subunit mRNA expression was reduced after repeated swim-stress in the mouse hippocampus (Montpied et al., 1993) and was reversibly increased in several brain regions after electroconvulsive shock (Kang et al., 1991). The latter finding corresponds with the observation of a brain region-specific, rapid, and transient change in $GABA_A$ receptor subunit expression after recurrent seizures (Kokaia et al., 1994) or of an increase in the number of postsynaptic $GABA_A$ receptor channels after chronic epilepsy induced by kindling (Otis et al., 1994; Titulaer et al., 1994). Again, the molecular mechanisms involved in the brain activity-dependent regulation of $GABA_A$ receptor expression and function so far have not been investigated.

VI. Conclusion

In conclusion, the heterogeneity of $GABA_A$ receptors in the brain is much larger than previously suspected. The considerable number of different $GABA_A$ receptor subunits present in the brain and their widespread but distinct regional distribution, the necessity for five subunits to assemble in order to produce intact $GABA_A$ receptors, and the recently collected evidence suggesting the occurrence of receptors containing either two, three, four, or possibly even five different subunits, indicate the existence of a multiplicity of $GABA_A$ receptors with different pharmacological and electrophysiological properties. The variable colocalization of different subunit mRNAs in individual cells and the different cellular and subcellular distribution and composition of receptors as determined by immunohistochemical techniques indicates that the composition of $GABA_A$ receptors actually is distinct not only in different parts of the brain but also in different cells or in the soma and dendrites of the same cell (Fritschy et al., 1992).

The composition of not a single $GABA_A$ receptor subtype is known so far. In situ hybridization and immunohistochemical investigations have, however, identified the tentative composition ($\alpha_1\beta_{2/3}\gamma_2$) of the most abundant $GABA_A$ receptor subtype in the brain. Receptors containing other subunit combinations seem to be less abundant. All available evidence, however, indicates that most, if not all, of the various subunit combinations investigated so far actually do exist in the brain and exhibit properties similar to those of their respective recombinant receptors. Recombinant receptors thus are convenient model systems for the investigation of the properties of individual $GABA_A$ receptor subtypes. In future studies, the pharmacological and electrophysi-

ological properties of GABA$_A$ receptors in intact brain tissue will have to be extensively compared with those of recombinant receptors with a defined subunit composition, in order to unambiguously identify the occurrence and localization of specific receptor subtypes expressed in the brain.

So far, it has not been possible to clarify which GABA$_A$ receptor subtypes mediate the various behavioural effects of GABAergic drugs. This is because no highly selective compounds interacting with a single GABA$_A$ receptor subtype are available, and even compounds with some selectivity exhibit a variable and largely unknown efficacy at receptors with a different subunit composition (see section III.B.4 of this article).

Recently, however, high affinity ligands have been developed for the diazepam-insensitive binding sites of Ro15–4513 (Wong and Skolnick, 1992; Gu et al., 1993) that probably are associated with the α_4- and/or α_6-subunit of GABA$_A$ receptors. Some of these compounds exhibited some selectivity for diazepam-insensitive over diazepam-sensitive sites, and one of these selective compounds has been synthesized in a radiolabeled form (Gu et al., 1992b). In addition, some of these high affinity ligands for the diazepam-insensitive sites could reproduce a discriminative stimulus in pigeons trained to Ro15–1788, and this effect could not be blocked by those high affinity agonists or antagonists at diazepam-sensitive receptors, which exhibited a low affinity for diazepam-insensitive sites (Wong et al., 1993). This finding for the first time might establish a link between a GABA$_A$ receptor isoform and a specific behaviour.

In other studies, diazepam-insensitive Ro15–4513 binding was absent from most of the benzodiazepine-sensitive alcohol-nontolerant rats (Uusi-Oukari and Korpi, 1990). A subsequent investigation identified a point mutation in the gene encoding the α_6-subunit of GABA$_A$ receptors in the alcohol-nontolerant rats that drastically enhanced the benzodiazepine agonist sensitivity of the GABA$_A$ receptors formed with the mutated subunit (Korpi et al., 1993). Instead of diazepam-insensitive receptors, the alcohol-nontolerant rats thus express receptors that can be potentiated by diazepam. These findings indicate that the excessive motor impairment caused by benzodiazepines in alcohol-nontolerant rats possibly reflects an anomalous response of their α_6-containing cerebellar GABA$_A$ receptors to these drugs. Thus, a specific phenotype possibly results from a point mutation in a GABA$_A$ receptor subunit. However, the role of α_6-containing GABA$_A$ receptors in cerebellar circuits that control motor reflexes, is not clear. In addition, although it has been speculated that the action of Ro15–4513, which antagonizes some of the behavioural effects of ethanol (Suzdak et al., 1986a; Lister and Nutt, 1987), is mediated by α_6-subunit-containing receptors (Lüddens et al., 1990), this claim so far has not been substantiated.

Studies, investigating the effects of gene knockouts by homogeneous recombination have not been published yet. A preliminary report, however, indicated that elimination of expression of the rather abundant and functionally important γ_2-subunit of GABA$_A$ receptors led to a lethal line of mice (Möhler et al., 1994). In contrast, it was demonstrated that mice that fail to express the γ_3- and the α_5-transcript were phenotypically normal (Culiat et al., 1994). Thus, the absence of the rather rare γ_3-subunit of the GABA$_A$ receptor alone, or the absence of both the γ_3- and the α_5-subunits, does not result in any overt neurological phenotype in mice. Additional studies, however, will be needed, to clarify whether there are any subtle developmental, behavioural, or pharmacological consequences of these deletions.

Because of the widespread use of GABA as a transmitter (Bloom and Iversen, 1971; Young and Chu, 1990), it is to be expected that modulation of GABA$_A$ receptors should influence a large number of different neuronal systems and should thus produce many behavioural effects in addition to the anxiolytic, anticonvulsant, muscle relaxant, and sedative hypnotic action induced by benzodiazepines. This conclusion is supported by evidence indicating that benzodiazepine receptor ligands are able to influence circadian rhythms (Turek and Van Reeth, 1988), appetite and food intake (Cooper, 1989), motor function, sexual reproduction and aggressive-defensive behaviours (Paredes and Agmo, 1992), and cognition, vigilance and memory (Roth et al., 1984; Duka et al., 1988; Sarter et al., 1988; Izquierdo and Medina, 1991).

Each of these effects of benzodiazepine receptor ligands presumably is produced in a different brain region. Thus, if it were possible to specifically address the subsets of receptors mediating these effects, highly selective behavioural actions could be expected. Depending on the desired effects, an enhancement as well as a reduction of the GABAergic transmission could have its therapeutic application. A selective enhancement of GABAergic transmission at the appropriate GABA$_A$ receptors should result in a separation of the anxiolytic from the sedative-hypnotic and atactic properties of the classical benzodiazepines or should offer the possibility of inducing presurgical anaesthesia without eliciting anterograde amnesia (Roth et al., 1984; Lister, 1985). A selective reduction of GABAergic transmission should enhance cognition, vigilance, memory, and learning without producing convulsions, anxiety, restlessness, and aggressive behaviour (Jansen, 1988).

Several allosteric binding sites (e.g., the benzodiazepine-, the steroid-, and possibly the γ-butyrolactone and the barbiturate sites) are available at the GABA$_A$ receptor, which could be used to induce a positive or a negative modulation of GABAergic transmission. It is to be expected that not only the properties of the benzodiazepine binding site but also those of the other allosteric binding sites at GABA$_A$ receptors depend on the receptor

composition. The investigation of the pharmacology of these allosteric binding sites in different recombinant receptors is therefore of considerable importance, because it might lead to the development of ligands selectively modulating a single $GABA_A$ receptor subtype.

So far, however, the benzodiazepine binding site is the only binding site at $GABA_A$ receptors for which at least some selective ligands have been identified (Sieghart, 1989; Stephens et al., 1990; Facklam et al., 1992a, b; Wong and Skolnick, 1992; Benavides et al., 1993; Gu et al., 1993). These ligands exhibit either a differential affinity for $GABA_A$ receptors or partial agonist properties. Thus, it has been demonstrated that for the induction of an anxiolytic, anticonvulsant, muscle relaxant, or sedative hypnotic effect, a different degree of $GABA_A$ receptor activation is necessary (Facklam et al., 1992a, b). For instance, full agonists are able to elicit an anxiolytic or anticonvulsant effect at a rather low overall receptor occupation. Partial agonists, because of their lower intrinsic efficacy for enhancement of GABAergic transmission, need a higher receptor occupancy to produce the same effect. To elicit sedative/hypnotic and muscle relaxant actions, however, even full agonists need a rather high receptor occupancy, and the weak enhancement of GABAergic transmission by partial agonists is not sufficient to induce these effects. Partial agonists, thus, have a more selective action and exhibit fewer side effects than full agonists (Facklam et al., 1992b).

The fact that partial agonists can exhibit selective behavioural effects, and the recent discovery that the efficacy of benzodiazepine receptor ligands for enhancing or reducing GABAergic transmission depends on receptor composition (Puia et al., 1991, 1992; Wafford et al., 1993a; Ducic et al., 1993), suggests another possibility to specifically address only certain $GABA_A$ receptors in the brain: compounds with a selective efficacy could be developed that enhance or reduce transmission at some, but not all, $GABA_A$ receptor subtypes. A modification of the chemical structures of antagonists, partial agonists, or partial inverse agonists might lead to such compounds, which could then be investigated in a series of recombinant receptors, representing the main $GABA_A$ receptor subtypes present in those brain regions where specific effects are desired. The investigation of the structure and pharmacology of $GABA_A$ receptor subtypes could thus open new avenues for the selective modulation of $GABA_A$ receptor subtypes in distinct brain regions.

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