Steroid Modulation of the GABA/Benzodiazepine Receptor-Linked Chloride Ionophore

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Abstract

Recent findings suggest that steroids with sedative-hypnotic properties interact specifically with the γ-aminobutyric acid$_A$/benzodiazepine receptor-chloride ionophore complex (GBRC). They show positive heterotropic cooperativity by allosterically enhancing the binding of GABA agonists and the clinically useful benzodiazepines (BZs) to their respective recognition sites. These steroids have stringent structural requirements for activity at the GBRC, with the essential requirements for high potency being a 3α-hydroxyl group and a 5α-reduced A-ring. Some of these steroids are naturally occurring metabolites of progesterone and deoxycorticosterone and have nanomolar potencies as potentiators of chloride channel conductance. These 3α-hydroxylated, 5α-reduced steroids do not act through any known sites on the GBRC. Thus, the exact site and mechanism of action remain to be determined. Together with the observation that physiological levels of these metabolites are sufficient to influence the function of the GBRC, the evidence clearly suggests a role for these steroids in the normal regulation of brain excitability by potentiating the postsynaptic effects of γ-aminobutyric acid (GABA). Pharmacological studies of the GBRC-active steroids show that they possess anxiolytic and anticonvulsant activities. The potential therapeutic application of these steriods in the treatment of mood disorders and catamenial exacerbation of seizures associated with the menstrual cycle is discussed. Collectively, the evidence from the studies of these steriods imply that another mechanism by which the endocrine system influences brain function has been identified. Its characterization will provide important insight into how steroids modulate brain excitability under normal and pathophysiological states.

Index Entries: Neuroactive steroids; 3α-hydroxylated, 5α-reduced steroids; γ-aminobutyric acid$_A$ (GABA$_A$) receptor-linked chloride ionophore; catamenial epilepsy; steroid regulation of brain excitability; barbiturates; GABA$_A$/benzodiazepine receptor complex; steroid modulation of the chloride ionophore; *t*-butylbicyclophosphorothionate labeled chloride ionophore; premenstrual syndrome.

Introduction

Steroid modulation of brain function and behavior via regulation of the genome is well known (Pfaff and McEwen, 1983). However, the ability of steroids to profoundly influence neuronal membrane excitability, and ultimately behavior, has been documented, but the site and mechanism(s) of action remain obscure. Progesterone and certain metabolites of progesterone have been known to produce sedative and anticonvulsant effects since the 1940s. (Selye, 1942). In addition, the anticonvulsant effect of these steroids is rapid and separable from their hormonal effects (Craig, 1966). Collectively, it is apparent that the evidence implies a direct action of these steroids on neuronal membranes. Based on the observation that certain progestins attenuate brain excitability, clinically useful steroid anesthetics were developed, most notably alphaxalone, which has been in clinical use until recently (Gyermek and Soyka, 1975). In retrospect, the observation that these steroid anesthetics have effects similar to those of the short-acting barbiturates on brain electrical activity provided the initial clue to their possible mechanism and site of action (Bellville, Howlands, and Bogan, 1956). In the interim, it has been established that the hypnotic barbiturates (e.g., Napentobarbital) produce these effects via interactions with γ-aminobutyric acid/benzodiazepine receptor-chloride ionophore complex (GBRC) in the brain. Alphaxalone was also found to potentiate GABA-mediated inhibition in the guinea pig olfactory (Schofield, 1980). It has now been demonstrated definitively that certain naturally occurring metabolites of progesterone can modulate the γ-aminobutyric acid$_A$ (GABA$_A$) receptor coupled chloride ionophore in a "barbiturate-like" manner (Majewska et al., 1986; Harrison et al., 1987). These findings have resulted in the hypothesis that these

steroids share a common site and mechanism of action with the hypnotic barbiturates.

The evidence that progesterone and certain naturally occurring progestins modulate brain excitability through the GBRC has profound implications when one considers the observation that GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Coupled with the in vitro observation that some of these progesterone metabolites have potencies even greater than clinically useful benzodiazepines (BZs) in modulating the GBRC, the evidence strongly supports the hypothesis that these steroids have an important physiological role in the regulation of brain excitability (Gee et al., 1987; Morrow, Sudzak, and Paul, 1987). The physiological implications of these findings have been recently reviewed (Majewska, 1987). The focus of this review will be on the possible site(s) and mechanisms(s) of action; the pharmacology and structure-activity requirements at the GBRC; and the potential therapeutic applications of these neuroactive steroids.

Functional Organization of the $GABA_A$/BZ Receptor Linked Chloride Ionophore

A detailed account of the molecular properties of this important receptor–ionophore complex are beyond the scope of this review and are documented elsewhere (Duman et al., 1987; Stephenson, 1988). However, to appreciate the nature of the complex interactions between steroids and the GBRC and their functional implications, an understanding of the pertinent features related to the functional organization of this macromolecular complex is essential. GABA influences neuronal excitability by interaction with a specific recognition site on the GBRC, which results in an increase in chloride ion conductance across the neuronal membrane (Bormann, Hamill, and Sakmann, 1987). Chloride ions flow down their electrochemical gradient to hyperpolarize the cell and render it generally less prone to excitation. There are at least three known drug binding sites on the GBRC that regulate the chloride channel either directly or indirectly through the $GABA_A$ receptor. Thus, GABAergic neurotransmission is highly regulated postsynaptically by various ligands that can allosterically modulate the recognition properties of the $GABA_A$ receptor.

The $GABA_A$ Receptor

The $GABA_A$ receptor in the mammalian CNS is the most well-characterized of multiple GABA receptors as defined pharmacologically. This receptor is part of a ligand-gated chloride ion channel (i.e., no evidence for second messenger involvement) and is activated by the naturally occurring ligand GABA. In contrast, the $GABA_B$ receptor, which also exists in the mammalian CNS, is coupled to one or more guanine nucleotide regulatory proteins, regulates K^+/Ca^{2+} channels, and has a different pharmacological specificity (Bowery et al., 1980; Dunlap, 1981; Hill, Bowery, and Hudson, 1984). It is not yet known whether steroids active at the GBRC will also interact with the $GABA_B$ receptor complex.

The $GABA_A$ receptor is defined pharmacologically by its specificity for the GABA antagonist (+)bicuculline and certain GABA agonists, including isoguvacine and piperidine 4-sulphonic acid (Enna, Collins, and Snyder, 1977; Bowery et al., 1981; Bowery, Hill, and Hudson, 1983). Binding studies with (+)bicuculline reveal the presence of low and high affinity binding sites. The relationship between these two states of the $GABA_A$ receptor is unclear. However, based on the micromolar quantities of GABA used to elicit a response in electrophysiological studies and in the modulation of BZ receptor binding, it appears that the low affinity site is the functionally relevant form of the receptor (Tallman, Thomas, and Gallager, 1978; Akaike, Maruyama, and Tokutomi, 1987).

It has been suggested that the high affinity form represents a desensitized state (Gallager, Rauch, and Malcolm, 1984).

Detailed electrophysiological studies in cultured spinal neurons demonstrate that GABA agonists can elicit the opening of the chloride channel with characteristic parameters, including channel conductance and mean channel open time (i.e., duration). All agonists induce similar changes in elementary conductance but different channel open times (Barker and Mathers, 1981). The ability to elicit different mean open times appears to be a function of agonist efficacy (i.e., partial vs full agonist). This type of analysis is used to determine the effect of allosteric modulators, including the steroids active at the GBRC in the presence or absence of GABA agonists. These studies also provide clues to the kinetics of chloride channel conductance and can be used to explain some of the complex binding kinetics of ligands that interact with the chloride channel (Gee, Lawrence, and Yamamura, 1986; Maksay and Simonyi, 1986). Thus, the effect of allosteric modulators on GABA-mediated responses can be measured at the electrophysiological and GABA recognition site levels and correlated with their pharmacological actions.

The Benzodiazepine Receptor

Many of the central pharmacological effects of the BZs are mediated by specific receptor sites that are integral parts of the GBRC. The BZ receptor on the GBRC is in part defined by its high affinity for the BZ compound clonazepam. Although there is heterogeneity of BZ recognition sites (e.g., the peripheral type specific for 4'-chlorodiazepam, also known as Ro5 4864), there is no evidence that steroids active at the GBRC will interact with these other BZ sites. The initial evidence for an allosteric link between the GABA and BZ receptors was provided by the observation that micromolar concentrations of GABA and related agonists could enhance the binding of certain BZs to the BZ receptor (Tallman, Thomas, and Gallager, 1978; Martin and Candy, 1978; Wastek et al., 1978). The enhanced binding is a result of increased affinity for the BZ receptor. The concentrations of GABA agonists required for enhancement coincide with those necessary to activate chloride channel conductance in electrophysiological studies. The latter observation lent additional evidence to the notion that the low affinity state of the $GABA_A$ receptor is the functionally relevant form. In addition, the rank order potency of GABA analogs as modulators of BZ binding and their ability to inhibit GABA binding were directly correlated (Falch et al., 1985). However, BZs alone do not have a direct effect on membrane conductance. The actions of BZs become detectable only when exogenously applied GABA is present or when GABAergic neurons are active (Haefely and Polc, 1982). Specifically, BZs increase the frequency of channel opening in response to GABA but have little effect on the mean duration of opening (Study and Barker, 1981; Choi, Farb, and Fishbach, 1981).

Subsequent to the identification of the BZ receptor by ligand binding assays, it was determined that compounds chemically unrelated to the BZs could also inhibit BZ receptor binding (Nielsen, Gredal, and Braestrup, 1979, Braestrup, Nielsen, and Olsen, 1980; Lippa et al., 1979). Notably, the methyl and ethyl esters of the β-carbolines interact with the BZs competitively, but have intrinsic activity opposite to that of the clinically useful BZs (Cowen et al., 1981; Braestrup et al., 1982). For example, methyl-β-carboline-3-carboxylate (MCC) has dose-dependent anxiogenic, proconvulsant, and convulsant activities (Braestrup et al., 1982). Another group of compounds represented by the imidoazodiazepine Ro15 1788 has little or no intrinsic activity but can antagonize the CNS effects of both the β-carbolines and the clinically used BZs at the BZ receptor (Hunkeler et al., 1981). Thus, the BZ receptor has the unique capability among drug receptors of mediating

opposing pharmacological actions, which raises the question of how these effects are mediated upon occupancy of the BZ receptor.

The ability of BZ receptor ligands to induce bidirectional changes in chloride channel conductance can be best understood within the framework of a two-state model of the GBRC, with each state corresponding to an open or closed channel conformation (Ehlert et al., 1983; Ehlert, 1986). The nature of the cooperative interactions has been previously reviewed in detail and only a summary will be presented here (Ehlert, 1986). The model proposes that the GBRC exists in equilibrium between two interconvertible states of the chloride ionophore, as shown in Fig. 1. The presence of distinct sites on the GBRC in addition to that for GABA allows for heterotropic (i.e., different types of sites involved) cooperativity between the sites found on the complex. The selectivity of the ligand will determine the direction of the equilibrium, with cooperativity between sites being positive or negative depending on whether a particular ligand will increase or decrease, respectively, the apparent affinity of another ligand. GABA binds selectively to the channel complex in the open conformation, thus allowing an increase in chloride conductance. The clinically useful BZs, such as diazepam, also have selectivity for the open conformation of the channel, but it is limited relative to GABA. As a result, they cannot induce conductance changes in the absence of GABA. However, these BZs will stabilize the complex in the open state induced by GABA. The reciprocal interactions resulting from this type of selectivity is demonstrated empirically by the observations that GABA will enhance the binding of diazepam, and binding to the $GABA_A$ receptor is enhanced by diazepam (Skerritt, Willow, and Johnston, 1982; Korneyev, 1983). Both effects are examples of positive heterotropic cooperativity. The degree of cooperativity between the binding of the drug and GABA to their respective sites determines the maximum effect of the drug. Thus, BZs such as diazepam have high therapeutic indices because of limited positive cooperativity. In contrast to diazepam, MCC has great selectivity for the closed conformation of the chloride channel and will display negative heterotropic cooperativity by inhibiting the binding of GABA to its recognition site. BZ receptor ligands that have little or no preference for either conformation of the channel complex will display no cooperativity and have no pharmacological activity on binding to either conformation. Ro15 1788 fits this profile and has been shown to block the effects of both the anticonvulsant BZs and MCC (Hunkeler et al., 1981; Braestrup et al., 1982). The aforementioned types of cooperativity also exist between the other sites present on the GBRC, including the recognition sites for barbiturates, picrotoxin, and the putative site for steroids (these will be discussed subsequently). Although the two-state model of the GBRC is most likely an oversimplification of the system, it nevertheless explains many of the cooperative interactions observed between steroids and the known recognition sites on this receptor–ionophore complex.

The Barbiturate Binding Site

Based on many of the shared pharmacological actions of the sedative-hypnotic barbiturates and the BZs, it is not surprising that these barbiturates may in part act through the GBRC. In contrast to the anticonvulsant BZs, barbiturates such as pentobarbital will enhance the actions of GABA by increasing the mean channel open time but have little effect on the frequency of channel opening in electrophysiological studies (Study and Barker, 1981). At higher concentrations (>100 μM), barbiturates will enhance chloride ion conductance in the absence of GABA (Jackson et al., 1982). The GABA enhancing effects are both stereospecific and chemically specific. Similarly, the same specificity is observed in vitro when barbiturate effects on binding to the $GABA_A$ receptor and the BZ

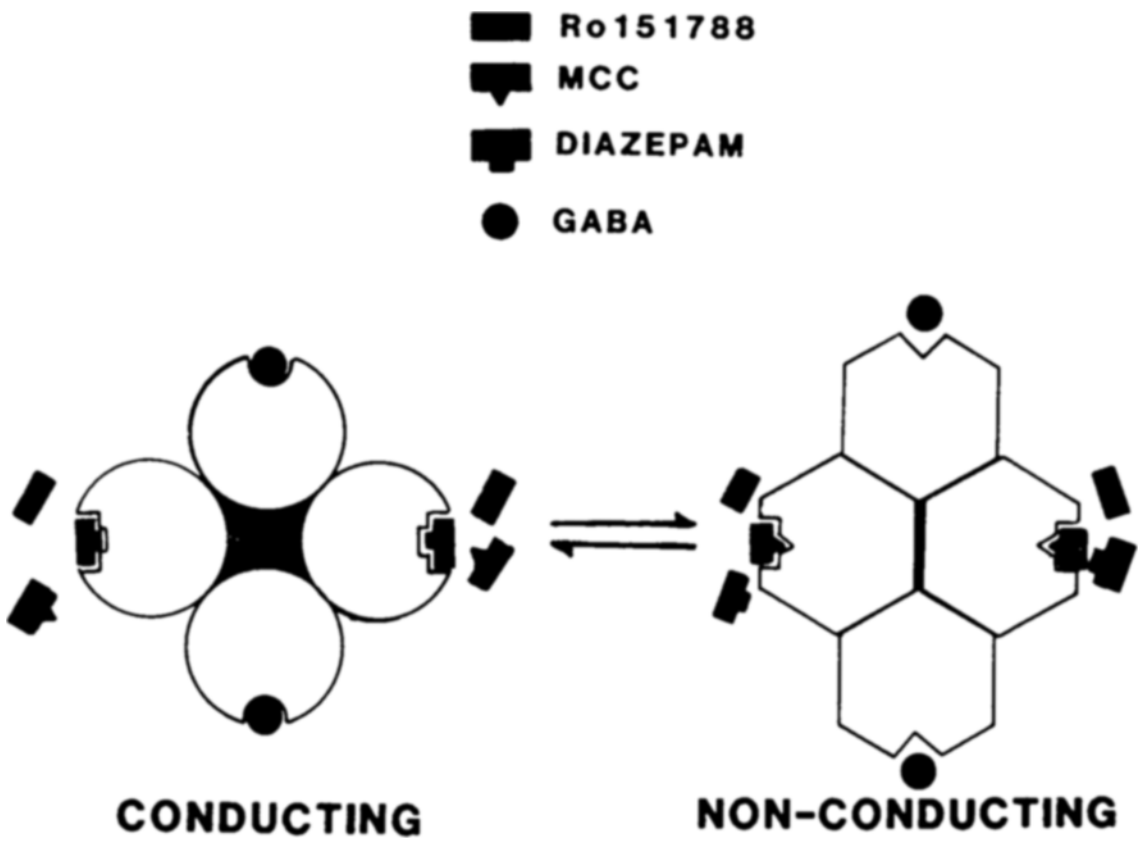


Fig. 1. A model for the GABA/benzodiazepine receptor–chloride ionophore complex. As shown, the GBRC exists in two interconvertible states associated with a conducting and nonconducting chloride channel. The open circles represent four transmembrane-spanning helices that form the ion channel (shaded region) in the open state. This conformation is stabilized by GABA and diazepam. In contrast, the helices undergo a conformational change (represented by open hexagons) in the presence of the β-carboline MCC causing the channel to close. Both the effects of MCC and diazepam can be blocked by the antagonist Ro15 1788, which will bind readily to either conformation.

receptor are measured. Direct binding of barbiturates to their recognition sites has not been performed because of their low affinity (K_d >100 μ*M*). However, indirect measurements indicate that the predicted allosteric interactions with the other recognition sites on the complex occur in accordance to the two-state model, and, further, support the intimate association of the barbiturate site with the GBRC. For example, barbiturates were found to enhance BZ receptor binding in a manner that correlates with their rank order potency as anesthetic/hypnotics (Leeb-Lundberg, Snowman, and Olsen, 1980). Similar specificities were observed for the rank order effect on the enhancement of $GABA_A$ receptor binding (Olsen et al., 1982). Both effects of the barbiturates involve enhancement of binding affinity.

The question of whether the barbiturate site is part of the same protein containing the $GABA_A$ and BZ receptors is of particular interest, since steroids active at the GBRC have been postulated to act at the same site as the hypnotic barbiturates (Majewska et al, 1986; Harrison et al., 1987). The observation that these barbiturates inhibit the binding of picrotoxin in the same

rank order of potency as hypnotics suggests a site of action near the chloride ionophore (Lee-Lundberg, Snowman, and Olsen, 1980). The convulsant picrotoxin is believed to be a non-competitive antagonist of GABA and acts by blocking chloride entry through the channel (Olsen, 1982). The importance of phospholipids in the allosteric modulation of BZ receptor binding by barbiturates is suggested by the observations that: phospholipase A_2 treatment will reduce barbituate induced enhancement of binding, and in CHAPS solubilized brain homogenates, cholesterol hemisuccinate will stabilize the enhancement of binding activity by barbiturates (Havoundjian et al., 1986; Bristow and Martin, 1987). Although these findings imply that the lipid environment contributes to the maintenance of native conformations of the GBRC that are conducive to normal function and perhaps to the integrity of the barbiturate recognition site, it does not determine whether the barbiturate site is part of the GBRC protein or the closely associated membrane lipid.

The Picrotoxin/ t-Butylbicyclophosphorothionate (TBPS) Binding Site

As alluded to earlier, picrotoxin is believed to antagonize the actions of GABA allosterically at a site closely associated with the chloride ionophore. Initial efforts to characterize this site with a radiolabeled analog of picrotoxinin was plagued with a low signal-to-noise ratio in binding studies (Ticku, Ban, and Olsen, 1978). More recently, [^{35}S]TBPS was found to label the same binding site recognized by picrotoxin but with much greater specificity (Squires et al., 1983). This cage convulsant will inhibit GABA-induced enhancement of chloride conductance in a manner identical to that of picrotoxin (Bowery, Collins, and Hill, 1976). Extensive structure-activity studies imply that compounds that bind to the picrotoxin/TBPS site and reduce chloride channel conductance do so by sterically hindering the entry of chloride across the ion channel (Klunk et al., 1983). The close proximity of this site to the chloride channel suggests that the binding of ligands such as [^{35}S]TBPS may be sensitive to the conductance state of the channel (i.e., open vs closed conformations). Thus far, the binding of [^{35}S]TBPS has been shown to be sensitive to all compounds that interact with the GBRC to modulate chloride channel conductance (Gee, Lawrence, and Yamamura, 1986; Maksay and Simonyi, 1986; Martin and Candy, 1978).

All compounds that are "GABA mimetics" (i.e., mimic or facilitate the effects of GABA) at the GBRC will inhibit steady-state [^{35}S]TBPS binding to its channel site in brain homogenates by reducing binding affinity. GABA inhibits [^{35}S]TBPS binding with micromolar potencies, lending additional support to the hypothesis that the low affinity GABA sites are functionally relevant. Hypnotic barbiturates will also inhibit [^{35}S]TBPS binding allosterically, whereas the convulsant barbiturates may inhibit binding directly (Squires et al., 1983). GABA "antagonists" (i.e., oppose the effects of GABA) such as the convulsant β–carbolines (e.g., MCC) will enhance [^{35}S]TBPS binding through specific interactions with the BZ receptor. In contrast, GABA mimetic BZs such as clonazepam will inhibit the binding of [^{35}S]TBPS but only in the presence of micromolar quantities of GABA (Lawrence, Gee, and Yamamura, 1984). The latter finding coincides with the observation that BZs affect membrane conductance to chloride only in the presence of GABA. Moreover, the BZs have limited efficacy relative to the hypnotic barbiturates and GABA agonists as inhibitors of steady state [^{35}S]TBPS binding (i.e., maximum inhibition less than that produced by GABA and the barbiturates). This limitation may be reflected in the inability of the BZs to induce anesthesia like the hypnotic barbiturates.

Detailed studies on the kinetics of [^{35}S]TBPS binding in the presence or absence of GABA mimetics and antagonists suggest that the bind-

ing site exists in two measurable conformations (Gee, Lawrence, and Yamamura, 1986; Maksay and Simonyi, 1986). GABA mimetics appear to allosterically shift the equilibrium in favor of a conformation with low affinity for TBPS, and the opposite occurs in the presence of GABA antagonists. It is postulated that the high affinity conformation is associated with a closed chloride channel, and GABA antagonists will stabilize the TBPS binding domain in the high affinity state. On the other hand, GABA mimetics inhibit steady-state TBPS binding by stabilizing the conformation with low affinity, for TBPS which is associated with the open state of the channel. The functional relevance of these phenomena is suggested by the finding that the rank order potency of various BZs as inhibitors of [^{35}S]TBPS binding is highly correlated with their potency as anticonvulsants against metrazol-induced seizures (Lawrence, Gee, and Yamamura, 1984).

Heterotropic cooperativity is also observed in interactions between the TBPS site and other sites on the GBRC (Gee, Lawrence, and Yamamura, 1986; Maksay and Simonyi, 1986; Gee, Brinton, and McEwen, 1988). For example, negative heterotropic cooperativity is demonstrated by the ability of TBPS to inhibit the binding of [^{3}H]flunitrazepam ([^{3}H]FLU) to the BZ receptor. This effect is consistent with the observation that each ligand is selective for opposite states or conformations (i.e., closed vs open states) of the GBRC. The binding of Ro15 1788 which has little preference for either of the two states of the GBRC, is unaffected by TBPS (Gee, Lawrence, and Yamamura, 1986). Thus, the nature of the allosteric interactions between the TBPS site and the other regulatory sites on the GBRC can be predicted by the two-state model. Additionally, the modulation of [^{35}S]TBPS binding can be used as a pharmacologically relevant response to drugs that can change chloride channel conductance via the GBRC. Ultimately, the development of fluorescent probes based on TBPS will allow kinetic measurements of the transition states of the chloride channel that correspond to those measured electrophysiologically by current fluctuation analysis under voltage-clamp (Study and Barker, 1981).

In addition to measuring the binding of [^{35}S]TBPS, a more direct method of evaluating chloride channel conductance in vitro has been used. Specifically, the flux of radiolabeled chloride ion (i.e., $^{36}Cl^-$) across synaptosomal preparations has been measured in response to GABA mimetics and antagonists (Schwartz et al., 1985; Obata el al., 1988). $^{36}Cl^-$ uptake by synaptoneurosomes is stimulated by GABA at micromolar concentrations and blocked by (+) bicuculline. The stimulation by GABA is enhanced by the hypnotic-barbiturates and the clinically useful BZs with all effects showing the appropriate pharmacology. Indeed, desensitization to the effects of GABA agonists can be also demonstrated in this preparation (Schwartz, Suzdak, and Paul, 1986). Collectively, the evidence suggests that measurement of $^{36}Cl^-$ flux in response to GBRC specific ligands may be a useful supplement to confirm findings obtained by electrophysiological measurements of chloride flux and [^{35}S]TBPS binding.

Steroid Modulation of the Chloride Ionophore

The initial evidence that certain steroids may have specific effects on GABA-mediated neurotransmission was provided by the observation that the steroid anesthetic alphaxalone (5α-pregnan-3α-ol-11,20-dione) enhanced GABA-stimulated chloride conductance in rat brain (Harrison and Simmonds, 1984). Recently, the 3α-hydroxylated, 5α-reduced metabolites of progesterone and deoxycorticosterone were found to allosterically modulate binding to the $GABA_A$/BZ receptors, and the TBPS site; and facilitate GABA stimulated uptake of $^{36}Cl^-$ by rat brain synaptoneurosomes

(Majewska et al., 1986; Harrison et al., 1987; Gee et al., 1987). All of these actions are consistent with their ability to enhance chloride channel conductance and to induce hypnosis/anesthesia. In addition, certain glucocorticoids and pregnenolone sulfate appear to have modulatory effects on the GBRC that are distinct from those of the 3α-hydroxylated, 5α-reduced steroids (Majewska and Schwartz, 1987; Ariyoshi and Akasu, 1986). The presence of a regulatory link between the hypothalamic-pituitary-adrenocortical (HPA) axis and the GBRC has been clearly demonstrated by the effects of adrenalectomy on BZ receptor binding in discrete brain regions (Goeders, De Souza, and Kuhar, 1986; De Souza, Goeders, and Kuhar, 1986; Miller et al., 1988). Coupled with the well-documented effect of the anxiolytic BZs on adrenal steroid hormone levels, and the effect of HPA hormones on behavior in animal models of anxiety, the possibility of the existence of an important feedback loop mechanism between the HPA axis and the GBRC can be rationalized (Lahti and Barsuhn, 1974). Perhaps the release of adrenocorticosteroids in response to stress ultimately results in the metabolism of these steroids into a form active at the GBRC, which in turn reduces stress and the subsequent release of corticosteroids. Clearly, investigations into the details of such a regulatory link will prove worthwhile. The subsequent discussion will focus on the possible mechanism(s) and site(s) of action by which the 3α-hydroxylated, 5α-reduced steroids modulate chloride channel conductance. Their effects will be explained within the confines of our current understanding of the functional organization of the GBRC.

Specific Modulatory Effects of Steroids on the GBRC

Initial demonstration of the in vitro interactions between 3α-hydroxylated, 5α-reduced steroids and the GBRC in rat brain revealed that they

1. Enhanced the binding of GABA agonists, such as muscimol at high nanomolar concentrations;
2. Enhanced [^{3}H]FLU binding;
3. Stimulated the uptake of $^{36}Cl^-$ into synaptoneurosomes; and
4. Inhibited the binding of TBPS to the chloride ionophore (Majewska et al., 1986; Harrison et al., 1987).

The electrophysiological data demonstrated that the "barbiturate-like" action of these steroids were also consistent with their in vitro effects on the GBRC. Collectively, these observations strongly support the hypothesis that the GBRC is a site of action for these steroids and that they have specific GABA mimetic-type effects. Certain other steroids, including pregnenolone sulfate and the glucocorticoids, have also been observed to interact with the GBRC but in a more complex manner (Majewska and Schwartz, 1987; Majewska, 1987).

The dose-dependent enhancement of muscimol binding in brain membrane homogenates by 5α-pregnane-3α-o1-20-one (3α-OH-DHP) most likely occurs by the enhancement of the binding affinity (Harrison et al., 1987). The observation that cholesterol also enhances the binding of muscimol raises questions of the relevance of measuring solely muscimol binding as a means to detect specific and functionally significant actions at the GBRC (Majewska, 1987). Moreover, it questions whether the effect of the 3α-hydroxylated, 5α-reduced steroids is a consequence of the interaction between the steroids and membrane phospholipids surrounding the GBRC (Fesik and Makriyannis, 1986). In order to help answer the former question, the functional consequences of cholesterol-enhanced muscimol binding was determined by measuring muscimol inhibition of [^{35}S]TBPS binding to rat cortical P_2 homogenates in the presence or absence of cholesterol. The underlying rationale of such an experiment is that muscimol should be a more potent inhibitor of [^{35}S]TBPS binding in the presence of cholesterol (10 μM) than in its absence. Under these conditions, we found that cholesterol has no effect on

the potency of muscimol as an inhibitor of [^{35}S]TBPS binding (unpublished observation). Thus, the enhancement of muscimol binding by cholesterol may not be a functionally significant effect. Furthermore, the presence of cholesterol at 0.1 m*M* had no effect on the binding of [^{35}S]TBPS. This observation suggests that cholesterol alters muscimol binding but not the conformation of the chloride channel (Gee et al., 1987). Consequently, the cholesterol effect may be peculiar to muscimol binding and is unrelated to the action of 3α-hydroxylated, 5α-reduced steroids.In contrast, the ability of 3α–hydroxylated, 5α-reduced steroids to enhance muscimol binding is fully consistent with their role as positive heterotropic modulators of the $GABA_A$ receptor. Another measure of the same effect is the observation that 3α-OH-DHP increases the potency of muscimol as a potentiator of [^{3}H]FLU binding to the BZ receptor (Harrison et al., 1987).

The stimulation of [^{3}H]FLU binding by steroids active at the GBRC also predicts the GABA mimetic effect of these steroids. The enhancement of binding observed occurs exclusively via an increase in the affinity for BZ receptor ligands with GABA-mimetic activity (Majewska et al., 1986). Interestingly, the EC_{50} (concentration at which half-maximal enhancement occurs) for 3α-OH-DHP enhancement of [^{3}H]muscimol binding is similar to the EC_{50} for the enhancement of [^{3}H]FLU binding (i.e., 100–170 nm), thus suggesting the effects are mediated by a common site (Harrison et al., 1987). The manner in which the $GABA_A$ and BZ recognition sites are modulated by 3α-OH-DHP is consistent with its postulated role as a naturally occurring potentiator of GABAergic neurotransmission in the CNS.

The measurement of steroid modulation of [^{35}S]TBPS binding has provided valuable insight into the possible mechanism(s) and site(s) by which these steroids exert their actions on GABA-regulated chloride channel conductance. The rank order potency of steroids as allosteric inhibitors of [^{35}S]TBPS binding coincide with their rank order as potentiators of GABA-stimulated chloride conductance in electrophysiological studies (Harrison et al., 1987). The utility of measuring [^{35}S]TBPS binding as a specific response to steroids and other allosteric modulators acting on the GBRC to modulate chloride channel conductance is well illustrated by the data in Fig. 2 (Gee et al., 1987). The inhibition of [^{35}S]TBPS binding to cortical P_2 homogenates by GABA is antagonized in a dose-dependent manner by (+)bicuculline. The inhibition curves shift to the right in a parallel fashion with increasing (+)bicuculline concentration. Schild analysis reveals that the antagonism is competitive with a slope of –1.05 and a K_B value of 4.8×10^{-7}*M* for (+)bicuculline (Arunlakshana and Schild, 1959). These observations are consistent with the known competitive interaction between GABA and (+)bicuculline at the $GABA_A$ receptor; and the K_B values agree with the K_d obtained by direct binding studies with [^{3}H](+)bicuculline (Mohler and Okada, 1978). In contrast, (+)bicuculline antagonizes the effect of the anesthetic steroid alphaxalone in a manner characteristic of an allosteric antagonist, as indicated by the slope of –1.46 derived by Schild analysis. The finding that 3α-OH-DHP enhances the binding of [^{3}H]muscimol to the $GABA_A$ receptor is also in agreement with the notion of an allosteric interaction (Harrison et al., 1987; Peters et al., 1988).

On measuring inhibition of [^{35}S]TBPS binding as a functional response to steroid modulation, it was determined that micromolar concentrations of GABA (5 μ*M*) will allosterically enhance the potency of steroids such as 3α-OH-DHP (Gee et al., 1987). This interaction is an example of reciprocal positive cooperativity between the $GABA_A$ receptor and the steroid binding site. Furthermore, in studies of 3α-OH-DHP stimulation of $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes, it was found that as little as 10 nm of these steroids will potentiate muscimol-stimulated $^{36}Cl^-$ uptake, thus confirming the studies demonstrating reciprocal cooperativity between the GABA and steroid

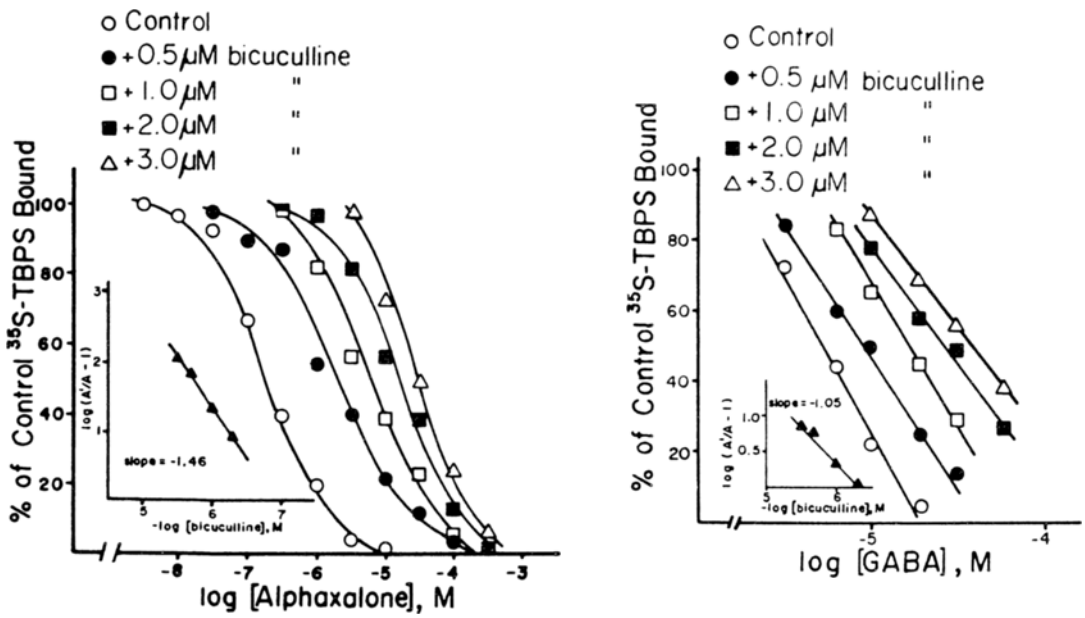


Fig. 2. The effect of (+)bicuculline on alphaxalone (left panel) and GABA (right panel) modulation of 2 nM [^{35}S]TBPS binding in rat cerebral cortex P_2 homogenates. Schild plots are in the inset to each panel with A = IC_{50} value in the absence of bicuculline (i.e., control) and A' = the IC_{50} value in the presence of bicuculline for alphaxalone or GABA under the respective conditions indicated. (O) Control; +bicuculline, 0.5 μM (•), 1.0 μM (□), 2.0 μM (■), 3.0 μM (Δ). Reproduced with permission from the European Journal of Pharmacology, Gee, K. W., Brinton, R. E., Chang, W. C. and McEwen, B. S. (1987), Gamma-aminobutyric acid-dependent modulation of the chloride ionophore by steroids in rat brain. *Eur. J. Pharmacol.* 136, 419–423.

sites against [^{35}S]TBPS binding (Morrow, Sudzak, and Paul, 1987). When compared to several clinically useful BZs, 3α-OH-DHP is more potent than the BZs in potentiating muscimol-stimulated $^{36}Cl^-$ uptake (Morrow, Sudzak, and Paul, 1987). The high nanomolar potencies of some of the steroids that are naturally occurring metabolites of progesterone and deoxycorticosterone strongly suggests the possibility that these metabolites play a physiological role in the regulation of GABAergic neurotransmission.

Mechanism(s) and Site(s) of Action

Several lines of evidence have been cited to support the hypothesis that the 3α-hydroxylated, 5α-reduced steroids and hypnotic barbiturates act on the GBRC through a common site and/or mechanism of action (Harrison et al., 1987). Electrophysiological studies using current and voltage clamp techniques in cultured spinal, hippocampal neurons, and bovine chromaffin cells indicate that like the barbiturates, 3α-OH-DHP and alphaxalone potentiate the effects of GABA by prolonging the open time of the chloride channel (Peters et al., 1988; Barker et al., 1987). Radioligand binding assays demonstrating the enhancement of [^{3}H]muscimol and [^{3}H]FLU binding and the inhibition of [^{35}S]TBPS binding by both the barbiturates and steroids have been proposed as additional evidence that they share a common mechanism of action (Harrison et al., 1987). Recently, additional studies on the hypothesis that 3α-hydroxylated, 5α-reduced steroids may act as "endogenous ligands" for the barbiturate recognition site on the GBRC have been performed (Gee et al., 1988). These studies have focused on the interactions between 3α-OH-DHP and Na pentobarbital as modulators of [^{3}H]FLU and [^{35}S]TBPS binding in rat brain.

The steady-state binding of [^{35}S]TBPS to the GABA-regulated chloride ionophor is inhibited by 3α-OH-DHP at nanomolar concentrations in the presence of micromolar concentrations of GABA (Gee et al., 1987). Kinetic studies reveal that maximally effective concentrations (i.e., produces 100% inhibition of steady-state [^{35}S]TBPS binding) of 3α-OH-DHP accelerate the dissociation but have little effect on association of [^{35}S]TBPS (Gee et al., 1988). This effect is produced by increasing the proportion of TBPS sites in the low affinity conformation (i.e., associated with the open conformation of the channel) without altering the rate constant for dissociation. Both 3α-OH-DHP and Na pentobarbital share this mechanism of action in modulating [^{35}S]TBPS binding. Thus, these steroids may enhance GABAergic inhibition by stabilizing the chloride channel in the open conformation.

The dissociation of [^{35}S]TBPS initiated by a saturating concentration of 3α-OH-DHP is potentiated by 100 μ*M* Na pentobarbital (a concentration that produces 90% inhibition of steady-state [^{35}S]TBPS binding, *see* Fig. 3). This effect is inconsistent with a common site of action for the steroid and barbiturate as inhibitors of [^{35}S]TBPS binding. Typically, allosteric modulators will alter the rate of dissociation of a radioligand initiated by a saturating concentration of a ligand that is a competitive inhibitor of the binding of the radioligand. Interactions between 3α-OH-DHP and Na pentobarbital in the potentiation of [^{3}H]FLU also result in behavior inconsistent with competition at a common site (Gee et al., 1988). Na pentobarbital has greater maximum efficacy than 3α-OH-DHP in the potentiation of [^{3}H]FLU binding in rat hippocampus. By examining the effect of varying amounts of 3α-OH-DHP on [^{3}H]FLU binding in the presence or absence of a maximally stimulating concentration of Na pentobarbital, the conclusion was reached that the two modulators do not potentiate [^{3}H]FLU binding via the same site (Fig. 4, *see* p. 14). Under the conditions used, 3α-OH-DHP should ultimately antagonize the effect of Na pentobarbital if the two interact competitively. In addition, 5β-pregnan-3α-o1-20-one will enhance the binding of [^{3}H]muscimol above that produced by a saturating concentration of secobarbitone in pig cerebral cortical membranes (Peters et al., 1988). This observation provides further evidence of distinct sites of action. Coupled with recent electrophysiological evidence demonstrating the potentiation of steroid activated transmembrane currents by barbiturates, it does not appear that these steroids and the barbiturates share a common site of action (Peters et al., 1988; Lambert, Peters, and Cottrell, 1987).

Interactions between several 3α-hydroxylated, 5α-reduced steroids as potentiators of [^{3}H]FLU binding show complex interactions suggesting distinct sites of action even among the steroids active at the GBRC (Gee et al., 1988). The highly lipophyllic nature of these steroids and the evidence that phospholipids are capable of binding steroids (e.g., alphaxalone) with great specificity raise the possibility that their effects are mediated by specific interactions with the membrane lipid–GBRC protein interface (Fesik and Makriyannis, 1986). On the other hand, the stringent structural requirements and the nanomolar potencies in the presence of GABA argue in favor of a specific action on the membrane protein. Studies on detergent solubilized membrane reveal similar stuctural requirements for the modulation of [^{35}S]TBPS binding and the presence of positive and negative heterotropic cooperativity (unpublished observations). Such studies predict a direct interaction with the GBRC unless these steroids perturb detergent micelles in a manner similar to their interaction with membrane phospholipids. Alternatively, steroids such as 3α-OH-DHP may produce their effects at nanomolar concentrations via direct interactions with the GBRC, whereas at micromolar concentrations their actions are the result of a membrane-disordering effect. The former effect is associated with the

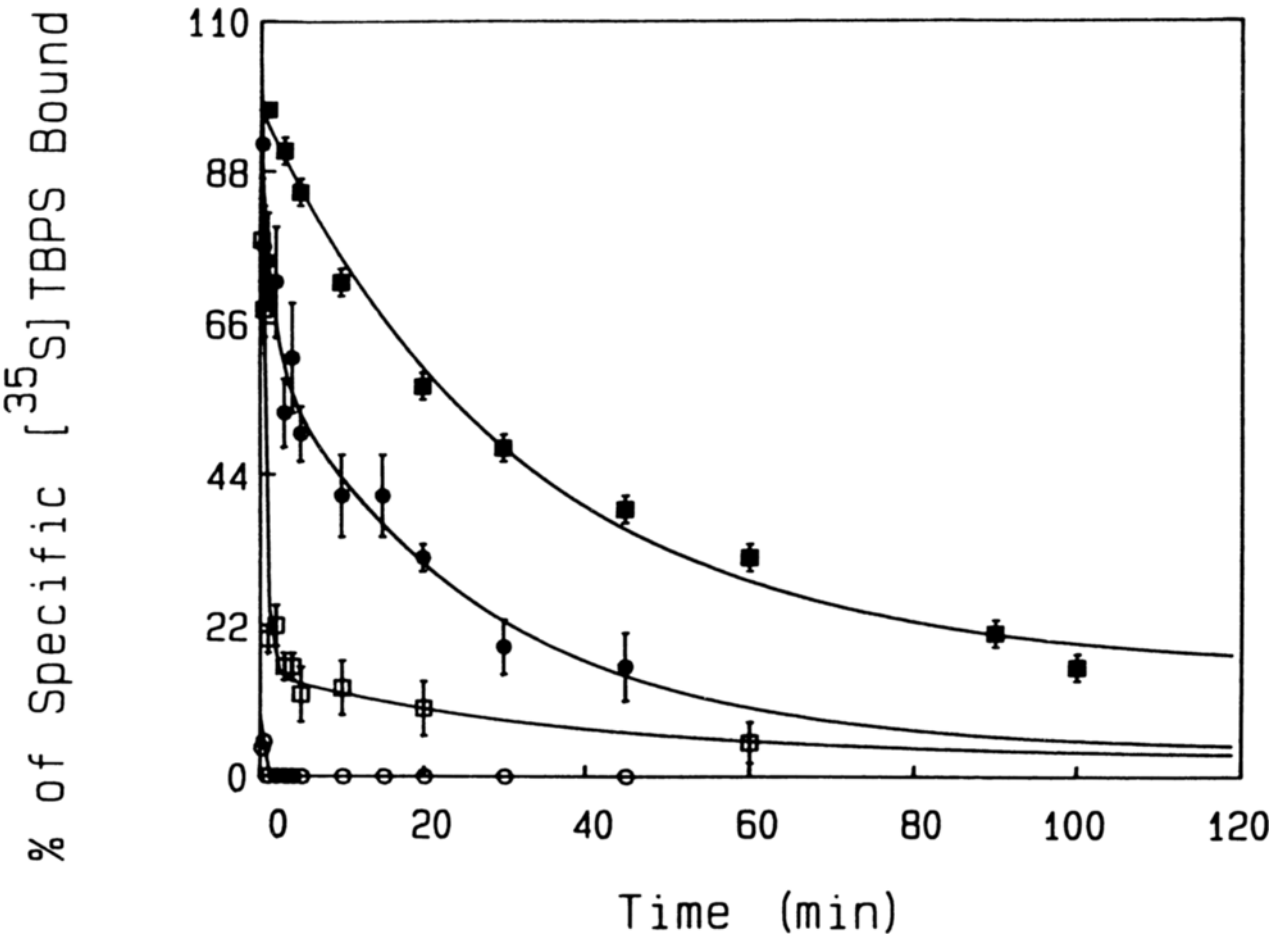


Fig. 3. Time course for the dissociation of 2 nM [^{35}S]TBPS from rat cortical P_2 homogenates initiated by the addition of 2 μ*M* TBPS (■), 1μ*M* 3α-OH-DHP (□), 100 μ*M* Na pentobarbital (•) and 1μ*M* 3α-OH-DHP + 100 μ*M* Na pentobarbital (•). All assays were performed in the presence of 5 μ*M* GABA at 25°C. Cortical P_2 homogenates were preequilibrated with [^{35}S]TBPS for 90 min prior to the initiation of dissociation. The abcissa is time (min) on a linear scale and the ordinate is the percent specific [^{35}S]TBPS bound at time zero. Each point represents the mean ± SEM of 4–11 independent determinations. Reproduced with permission from the Journal of Pharmacology and Experimental Therapeutics, Gee, K. W., Bolger, M. B., Brinton, R. E., Coirini, H. and McEwen, B. S. (1988), Steroid modulation of the chloride ionopohore in rat brain: structure activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**, 803–812.

potentiation of GABA, whereas the latter effect may underlie the anesthetic effect of the steroid. Indeed, recent observations that 3α-OH-DHP is 3× more potent in enhancing muscimol-stimulated $^{36}Cl^-$ uptake than in directly stimulating $^{36}Cl^-$ uptake, and 10× more potent in the potentiation of GABA than in direct activation when based on electrophysiological data has resulted in the suggestion that the potentiation of GABA inhibition is the physiologically relevant event (Morrow, Sudzak, and Paul, 1987; Barker et al., 1987). Nevertheless, the question of biological significance of these steroids in vivo awaits the development of specific antagonists. Such antagonists will provide the ability to determine whether these steroids modulate GABA function in vivo. Alternatively, selective alteration of the synthesis of these steroids, thus altering

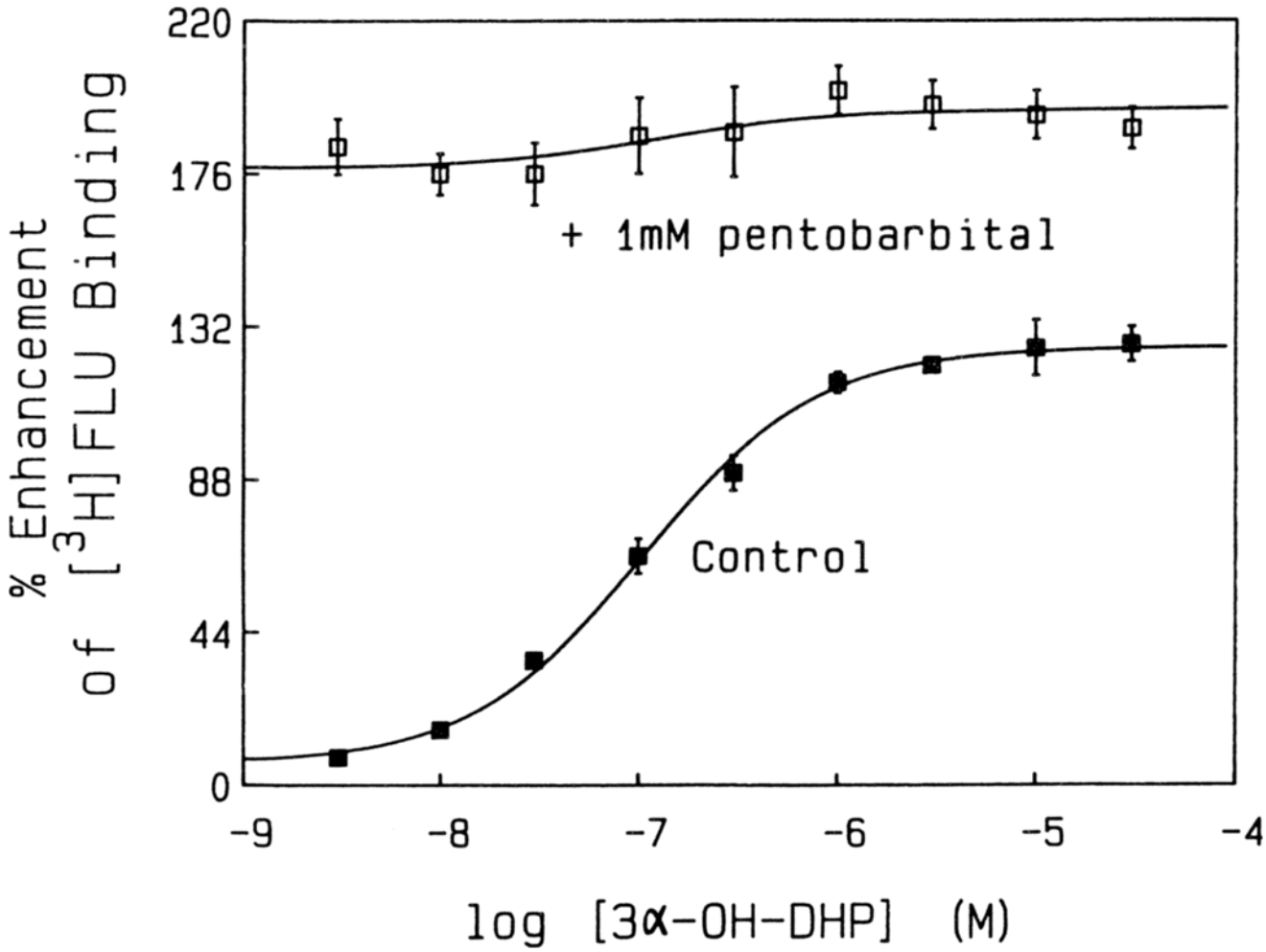


Fig. 4. Effect of 1 m*M* pentobarbital on 3α-OH-DHP enhancement of 0.25 n*M* [^{3}H]FLU binding in rat hippocampal P_2 homogenates. Each point represents the mean ± SEM of 4–6 independent determinations and is expressed as percent enhancement of [^{3}H]FLU binding (defined as the percentage of [^{3}H]FLU bound in the absence of any modulator minus 100%). All assays were performed at 25°C in the absence of GABA. Reproduced with permission from the Journal of Pharmacology and Experimental Therapeutics, Gee, K. W., Bolger, M. B., Brinton, R. E., Coirini, H. and McEwen, B. S. (1988), Steroid modulation of the chloride ionophore in rat brain: structure activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**, 803–812.

their levels, may also provide clues as to their biological significance.

The recent isolation of cDNAs encoding the $GABA_A$ receptor polypeptides (i.e., α and β subunits) will permit definitive answers to the question of which subunit of the receptor–ionophore oligomer is the site through which neuroactive steroids modulate the receptor complex and the relative importance of membrane lipids to their action (Schofield et al., 1987). The possibility of a recognition site for steroids on the GBRC is further reinforced by the findings that 3α-OH-DHP will enhance [^{3}H]FLU binding to the soluble BZ receptor purified to homogeneity

by affinity chromatography (unpublished observation). Coexpression of the respective cDNAs in cell lines or yeast will yield large amounts of receptor protein for the determination of the presence of cooperative interactions between the known recognition sites and the steroid site. The functional expression of the $GABA_A$ receptor by microinjection of pure subunit RNAs into the *Xenopus* oocyte will determine whether steroid modulation of a GABA induced response is present in the oocyte membrane (Schofield et al., 1987). The combination of these two powerful techniques in site-directed mutagenesis studies will yield detailed information on the location of the steroid site relative to the other recognition sites and the chloride channel.

Direct studies on the regional distribution of steroid-regulated chloride channels in rat brain indicate that 3α-OH-DHP has regionally dependent differences in potency (Gee et al., 1988). For example, the rank order poteny of 3α-OH-DHP as a modulator of [^{35}S]TBPS binding is hippocampus ≃ cerebellum > cortex. Thus, it is reasonable to assume that these steroids may have regionally selective effects based on dose. These differences in potency do not appear to be the result of regional differences in sensitivity to GABA (i.e., GABA always enhances the potency of 3α-OH-DHP). The possible influences of other regulatory factors affecting steroid potency have not been ruled out. Autoradiographic studies demonstrate that all [^{35}S]TBPS labeled sites in rat brain appear functionally coupled to a steroid site mediating the effects of 3α-OH-DHP (Fig. 5). These studies do not rule out, however, the possibility that TBPS and steroid sites exist independently and the presence of a heterogenous population of steroid binding sites. Interestingly, $GABA_A$ receptor-medated contractile responses in the guinea pig ileum are modulated biphasically by cortisol (Ong, Kerr, and Johnston, 1987). Thus, steroid recognition sites with different specificities appear to be coupled to both central and peripheral $GABA_A$ receptors.

Recently, pregnenolone sulfate, a steroid found in the brain, was reported to act as a "picrotoxin-like" antagonist at the GBRC (Majewska and Schwartz, 1987). It was proposed that pregnenolone sulfate acts at the TBPS site through which it inhibits pentobarbital enhanced BZ binding and GABA agonist-stimulated ^{36}Cl uptake into synaptoneurosomes. At the same time this steroid has been reported to enhance muscimol and BZ binding (Majewska, Bisserber, and Eskay, 1985). Unless this steroid has multiple sites of action or partial agonist properties at a single site, the combination of opposing actions appears anamolous, especially since it was proposed that pregnenolone sulfate is a "natural ligand" for the TBPS site (Majeweska and Schwartz, 1987).

Detailed evaluation of the intriguing hypothesis that pregnenolone sulfate is a ligand for the TBPS site has resulted in several discrepancies (Gee, Joy, and Belelli, in press). In vivo, pregnenolone sulfate will significantly increase the time to onset of myoclonus induced by TBPS (.025 mg/kg) in mice, an apparent anticonvulsant effect (Gee et al., 1988). In vitro, the dissociation of 2 n*M* [^{35}S]TBPS from rat cortical homogenates initiated by excess TBPS (i.e., a supersaturating concentration) is allosterically modulated by pregnenolone sulfate (Gee, Joy, and Belelli, in press). The effect of pregnenolone sulfate is to accelerate the dissociation of [^{35}S]TBPS, an effect that is inconsistent with a competitive interaction at the TBPS site. Furthermore, the potency of pregnenolone sulfate as an inhibitor of [^{35}S]TBPS binding is enhanced in the presence of micromolar quantities of GABA (Gee, Joy, and Belelli, in press). Thus, the apparent positive heterotropic cooperativity displayed does not coincide with the proposed "TBPS-like" activity of pregnenolone sulfate, but is instead consistent with its apparent anticonvulsant effect (Gee et al., 1972). Studies on the interactions between 5α-pregnanediol and pregnenolone sulfate as modulators of [^{35}S]-TBPS and [^{3}H]FLU binding (unpublished data) in rat cortical homogenates suggest that the two

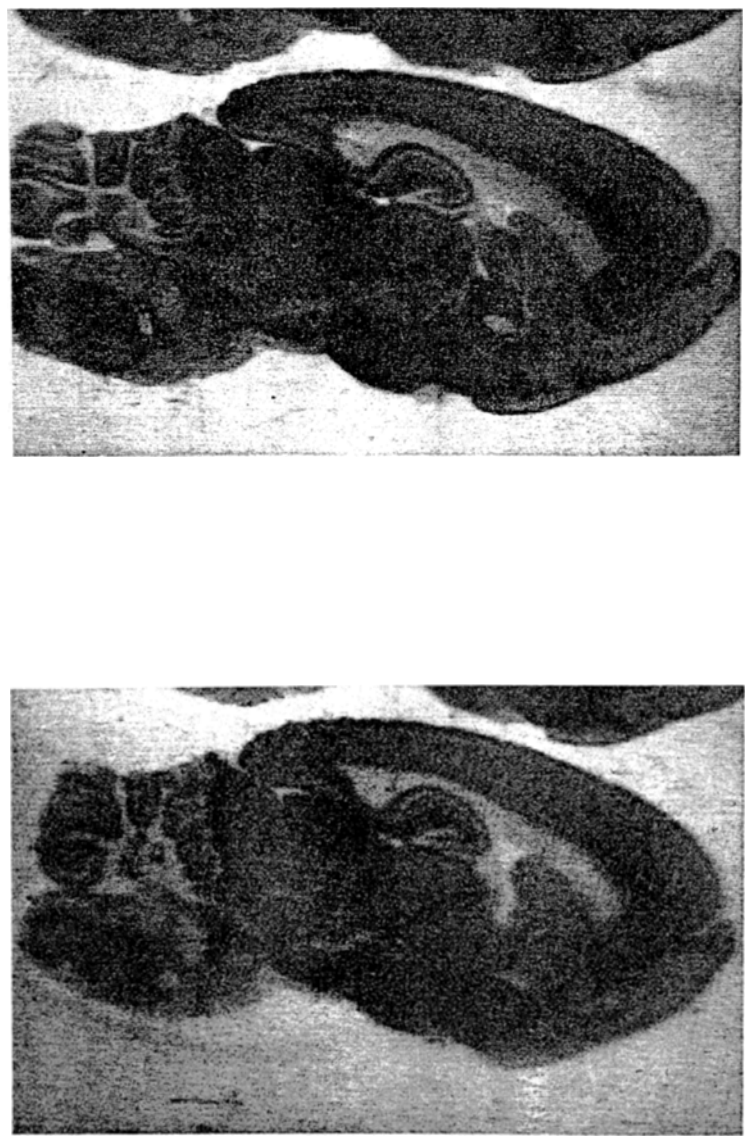


Fig. 5. Regional distribution of 10 nM [^{35}S]TBPS labeled sites in saggital sections of rat brain in the presence (lower panel) or absence (upper panel) of 30 nM 3α-OH-DHP. Serial 16 μm brain section were incubated in the presence or absence of 30 nM 3α-OH-DHP and 10 nM [^{35}S]TBPS at 25°C for 90 min. All assays were carried out in the presence of 5 μ*M* GABA with nonspecific binding being defined as binding in the presence of 10 μ*M* TBPS. Nonspecific optical density was uniform throughout the brain and across conditions.

types of steroids do not share a common site of action at the GBRC (Gee, Joy, and Belelli, in press). These observations provide additional evidence for the complexity of the interactions between steroids and the GBRC.

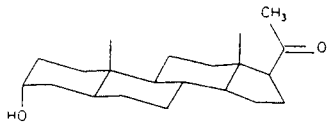
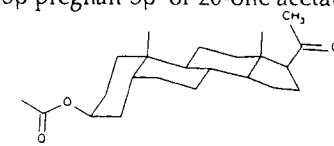
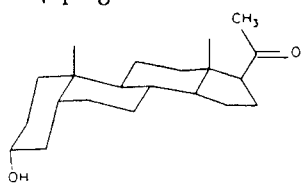
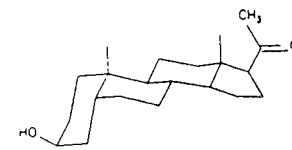
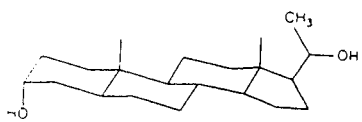
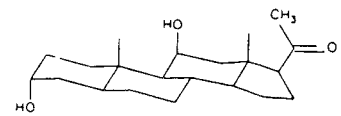
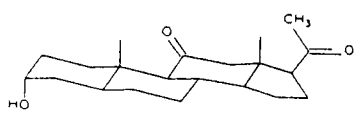
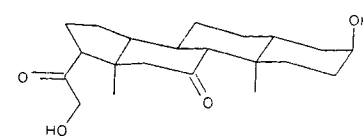
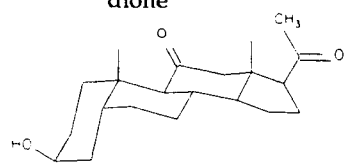
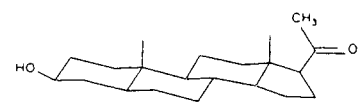
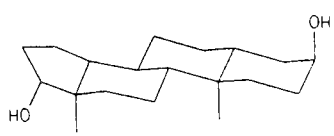
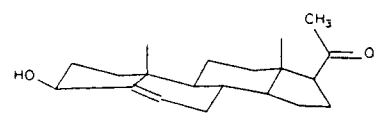
Pharmacology of Steroids Active at the GBRC

Most of the early CNS pharmacological studies on steroids that are now known to be active at the GBRC were focused on the evaluation of their sedative–hypnotic and anesthetic properties (Gyermek and Soyka, 1975; Bellville, Howland, and Bogan, 1956; Schofield, 1980). For example, it was observed that progesterone required 5–15 min for the onset of hypnotic activity, implying that the steroid must first be metabolized (Figdor et al., 1957). Although progesterone has weak activity at the GBRC (Gee et al., 1987), the central pharmacological effects are most likely mediated by the 3α-hydroxylated, 5α-reduced metabolites (Smith, Waterhouse, and Woodward, 1987a,b). It has been known for some time that ring A reduced steroids have little or no steroid hormonal activity (Dorfman and Ungar, 1965), and is best illustrated by the inability of two of the most potent GBRC-active steroids, 3α-OH-DHP and 5α-pregnan-3α,21-diol-20-one (THDOC), to inhibit the binding of the progestin [^{3}H]R5020 (promegestrone) to the cytosolic progesterone receptor from rat uterus (Gee et al., 1988). This specificity of action coupled with the knowledge of the structure-activity requirements for interaction with the GBRC encourages further evaluation of the CNS pharmacology of these steroids. Earlier studies of this type resulted in the development of steroid anesthetics that were used clinically until recently (Gyermek and Soyka, 1975). Perhaps the new studies will lead to the identification of novel steroids that can be used to selectively modulate brain excitability via the GBRC.

Structure–Activity Requirements for Potency and Efficacy at the GBRC

Structural requirements for activity at the GBRC are shared between two endpoints (i.e., modulation of [^{35}S]TBPS binding and Cl^- channel conductance). Since the same requirements are necessary for hypnotic and anesthetic activity, it implies that the mediation of these actions occurs via the GBRC (Harrison et al., 1987; Gee et al., 1988). The two key features necessary for activity against [^{35}S]TBPS binding include a 5α- or 5β-reduced steroid A-ring, and a 3α-OH group (Table 1). Even androstanes in the 5α,3α-ol configuration are fully active but with lower potency when compared to the pregnanes. The 3α-ol steroids with a 5α configuration have greater potency than those that are 5β-reduced. Stringent stereoselectivity requirements are demonstrated by the observation that the 5α,3α-ol is 6000× more potent than the 5α,3β-ol. The reduction in both potency and efficacy following the introduction of a 3β-OH is most pronounced in the 5α series. The introduction of a ketone at the C-11 position diminishes potency in the 5α,3α-ol series but produces the opposite effect in the 5β,3β-ol series. Reduction of the C-11 ketone to a β-hydroxyl results in a pronounced decrease in potency (~7.5×) in the 5α,3α-ol series (i.e., alphaxalone vs 5α-pregnan-3α, 11β-diol-20-one). The addition of a hydroxyl group at C-17 results in the loss of activity against [^{35}S]TBPS binding (Harrison et al., 1987). The presence of a C-21 hydroxyl will lower the potency against [^{35}S]TBPS binding in the 5α, 3α-ol series (Gee et al., 1987). Similar structure-activity requirements are observed when evaluating the rank order potency of these steroids as modulators of TBPS-induced convulsions in mice (Gee et al., 1988). These steroids produce a dose-dependent reduction in the time to onset of myoclonus induced by a CD^{99} (i.e., dose that produces convulsions in 99% of animals tested) dose of TBPS in mice.

Table 1
Allosteric Modulation of [^{35}S] TBPS Binding to Rat Cortcal P_2 Homogenate by Various Steroids[a]

Compound Name	Maximal Inhibition %	IC_{50}nM	Compound Name	Maximal Inhibition %	IC_{50}nM
5α-pregnan-3α-ol-20-one 	100	17 ± 3	5β-pregnan-3β–ol-20-one acetate 	71	1044 ± 85
5β-pregnan-3α-ol-20-one 	100	61 ± 11	5β-pregnan-3β-ol-20-one 	73	1739 ± 654
5α-pregnan-3α, 20-diol 	52	82 ± 11	5α-pregnan-3α-11β-diol-20-one 	100	2000 ± 360
5α-pregnan-3α-ol-11, 20-dione Alphaxalone 	100	264 ± 49	5α-pregnan-3α,21-diol-11, 20-one 	100	5500 ± 475
5β-pregnan-3β-ol-11, 20-dione 	67	487 ± 85	5α-pregnan-3β-ol-20-one 	33	>10^5
5α-androstan-3α,17β-diol 	100	1060 ± 85	5-pregnan-3β-ol-20-one 	30	>10^5

[a]All assays were performed in the presence of 5μ*M* GABA. All IC_{50} values (concentration of steroid that produces half maximal inhibition of [^{35}S] TBPS binding) represent the geometric mean ± SEM of 3–4 determinations.

The most interesting structural modification as it relates to alterations in efficacy is that produced by the reduction of the C-20 ketone in 3α-OH-DHP resulting in 5α-pregnan-3α,20α-diol (5α-pregnanediol). This steroid has no activity against [^{35}S]TBPS binding in the absence of GABA. However, when GABA is present, 5α-pregnanediol has limited efficacy but high potency in modulating [^{35}S]TBPS binding. The effect on [^{35}S]TBPS binding is reminiscent of the effect of BZs such as clonazepam under similar conditions (Fig. 6). These findings suggest that like the BZs, this steroid is active only in the presence of GABA and may exert a limited degree of positive cooperativity on the $GABA_A$ receptor which is reflected in its limited efficacy in modulating [^{35}S]TBPS binding to the ion channel (Gee et al., 1987). Thus, steroids like 5α-pregnanediol may possess the desirable properties of the BZs including high potency and a high therapeutic index. Notably, this steroid is as potent as clonazepam against [^{35}S]TBPS and ten times more potent than diazepam and flurazepam in potentiating $GABA_A$ receptor mediated ^{36}Cl uptake in synaptoneurosomes (Morrow, Sudzak, and Paul, 1987; Gee et al., 1988). When compared to 3α-OH-DHP, it is tempting to speculate that 5α-pregnanediol may be a partial agonist at a common site of action.

The early pharmacological evaluation of the 3α-hydroxylated, 5α-reduced steroids focused almost exclusively on their sedative-hypnotic potential. With the recent evidence that these steroids exert these effects through the GBRC, the evaluation of other pharmacological properties associated with activity at GBRC (i.e., anticonvulsant, anxiolytic activities) have been performed (Backstrom, Bixo, and Hammarback, 1985; Crawley et al., 1986). The deoxycorticosterone metabolite, THDOC, has been evaluated in 2 animal models of anxiety: the two-chambered exploration test and the lick-suppression conflict test. The former test is based upon the spontaneous tendency of mice to prefer a small dark chamber over an open and brightly lit one (Crawley and Goodwin, 1980; File, 1980). Anxiolytics will increase the frequency of exploration into the brightly lit and open chamber. The lick-suppression test uses a noxious stimulus (i.e., electric shock) to suppress an appetitive behavior such as drinking. Enhancement of suppressed H_2O consumption is characteristic of anxiolytics (Vogel, Beer, and Clody, 1971). THDOC (5–15 mg/kg ip) was found to have anxiolytic effects in both tests in the absence of any confounding actions such as stimulant or sedative effects (Crawley et al., 1986). Furthermore, the magnitude of the anxiolytic effect was similar to many of the clinically used BZs.

The anticonvulsant effects of 3α-hydroxylated, 5α-reduced steroids have been evaluated in a number of seizure models. 3α-OH-DHP administered systemically will rapidly suppress epileptic spikes produced by penicillin foci in cats (Backstrom, Bixo, and Hammarback, 1985). Progesterone will also produce a similar effect but is 200× less potent, a difference that compares favorably to the difference in potency of the two steroids as inhibitors of [^{35}S]TBPS binding (Harrison et al., 1987). 3α-OH-DHP is effective against metrazol-induced seizures in mice with an ED_{50} of 18 mg/kg ip (unpublished observation). The anticonvulsant effect of the 3α-hydroxylated, 5α-reduced steroids against TBPS induced convulsions was described earlier. Using the kindling model of epilepsy, alphaxalone and alphadione were found to inhibit the development of seizures in immature rats (i.e., 30 d old) by preventing the generalization of kindled seizures (Holmes et al., 1987). Interestingly, this steroid had little effect on seizure development in mature (i.e., 8–10 wk old rats). This observation appears to correlate with the finding that alphadione is highly toxic to neonatal rats (Gyermek, 1974). The toxicity appears peculiar to hypnotic steroids since a number of other anesthetic agents do not have selective toxicity in newborn animals. It is tempting to speculate that this phenomenon may be a reflection of developmental changes in steroid site-GBRC coupling or the steroid site itself.

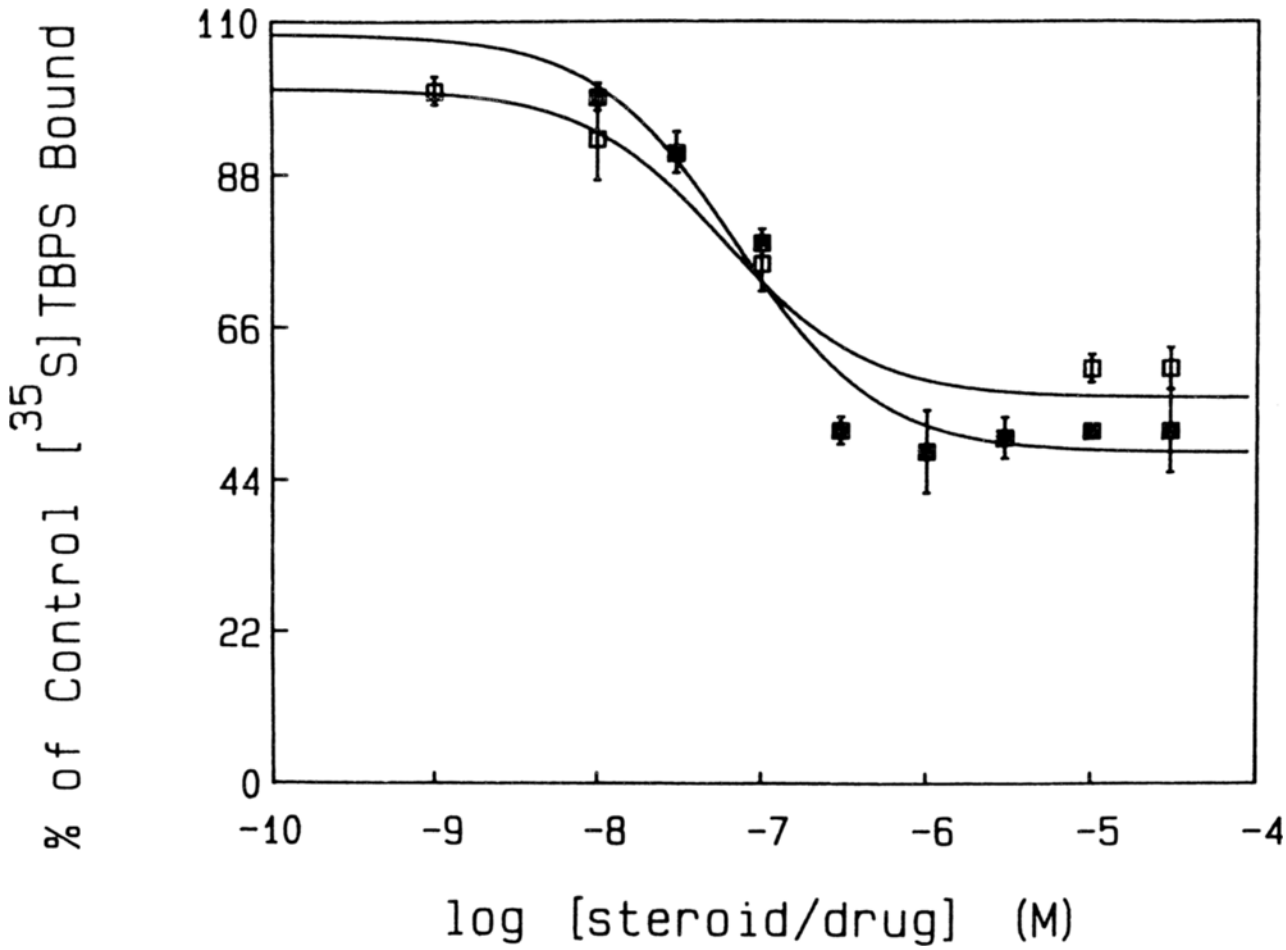


Fig. 6. Dose-dependent inhibition of [^{35}S]TBPS (2 nM) binding by 5α-pregnan-3α,20-diol (■) or clonazepam (□) in rat cerebral cortical P_2 homogenates. All assays were run at 25°C, 90 min in the presence of 5 μ*M* GABA. Each point represents the mean ± SEM of 3–4 independent determinations. The IC_{50} (concentration that produces half-maximal inhibition of [^{35}S]TBPS binding) values (geometric mean ± SEM) for 5α-pregnan-3α,20-diol and clonazepam were 82 ± 11 nM and 93 ± 29 nM, respectively. Reproduced with permission from the Journal of Pharmacology and Experimental Therapeutics, Gee, K. W., Bolger, M. B., Brinton, R. E., Coirini, H. and McEwen, B. S. (1988), Steroid modulation of the chloride ionophore in rat brain: structure activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**, 803–812.

Potential Therapeutic Applications

Two potential therapeutic applications of 3α-hydroxylated, 5α-reduced steroids may ultimately result from studies on the relationship between brain excitability and cyclic changes in the levels of the naturally occurring forms of these steroids. The disorders of particular interest are premenstrual syndrome (PMS) and catamenial epilepsy. The former syndrome is a symptom complex of unknown etiology with anxiety and stress being the predominant emotional symptoms (Dalton, 1984). The adverse changes in mood are reported to peak within the last 5 premenstrual days (Backstrom, Bixo, and Hammarback, 1985). In a similar fashion, women with catamenial epilepsy associated with menses report increased seizure incidence at premenses (Backstrom, Bixo, and Hammarback, 1985; Newmark and Penry, 1980). Collec-

tively, the extensive clinical data suggests that seizure susceptibility is best correlated with the plasma level of progestins and the estrogen/progesterone ratio (Newmark and Penry, 1980). During periods of high progesterone levels seizure incidence is low and vice versa. Clinically, the synthetic progestins, norethisterone and medroxyprogesterone acetate, were found to be active in the treatment of catamenial epilepsy (Newmark and Penry, 1980). Considering the low potency of progesterone at the GBRC, perhaps metabolites of progesterone active at the GBRC may be more effective than progesterone and its congeners. A recent clinical study in 64 female epileptic patients found a correlation between reduced progesterone metabolite (i.e., 5α and 5β pregnanediol) and increased seizure frequency, but no correlation with increased estrogen levels (Rosciszewska et al., 1986). Animal studies are also suggestive of similar relationships between GBRC-active progesterone metabolites and seizure susceptibility. For example, during the rat estrus cycle, cyclic changes in the seizure threshold are correlated with levels of these metabolites. Susceptibility to electroshock seizures is highest during estrous and proestrous, periods during which levels of 3α-OH-DHP and 5α-pregnanediol are lowest (Wooley and Timiras, 1962; Holzbauer, 1975). The plasma levels of these progesterone metabolites are sufficient to influence the GBRC (Holzbauer, 1975). The overall evidence plus the observations that the enzymes necessary for the production of GBRC-active progesterone metabolites are present in the brain and periphery is strongly suggestive of a role for these steroids in the modulation of brain excitability (Karavolas et al., 1984). The relative importance of their role will be determined, in part, by measuring the effect of selectively altering the brain levels of these metabolites on seizure susceptibility.

The complexity of the behaviors associated with PMS makes it difficult to evaluate systematically and assign primary responsibility for any particular symptom to a single underlying biochemical disturbance. However, the observation that a precipitous drop in the levels of progesterone coincides with the onset of stress and anxiety provides at least a starting point to evaluate a possible biochemical basis for the emotional symptoms. The observation that only progesterone itself is effective in cases where relief from PMS is observed, and that in some cases progesterone is not effective, suggests the possibility that the GBRC-active progesterone metabolites may be the effective agent (Dalton, 1984). The former observation may be related to the inability to metabolize synthetic progestins to 3α-OH-DHP and other active forms, thus explaining the lack of activity of the synthetics. In cases where progesterone is ineffective in relieving stress and anxiety, it may be a result of a diminished capacity to convert progesterone to GBRC-active metabolites. Such speculations can only be substantiated by measuring the plasma levels of these active metabolites during the menstrual cycle. A sudden and precipitous drop in these metabolites prior to menses may underlie the symptoms of anxiety and stress associated with PMS. One possible therapeutic regimen may be to administer these naturally occurring "anxiolytic" steroids immediately prior to the drop in plasma levels and during the subsequent premenstrual period. From a practical standpoint, the use of such steroids is not possible because of the susceptibility of the 3α-OH groups to rapid metabolism and subsequent excretion. Moreover, the efficacy of 3α-OH-DHP and THDOC at the GBRC is similar to that of the hypnotic barbiturates and, thus, they share the adverse side effects that limit the use of barbiturates as anxiolytics and anticonvulsants. Nevertheless, the possible existence of steroids with partial agonist properties (i.e., 5α-pregnanediol) at the GBRC encourages further investigations aimed at identifying steroids with therapeutic potential in similar applications but with limited side effects.

A preliminary observation of potential importance is the recent finding that 3α-OH-DHP produces dose-dependent analgesic responses in male mice that are stereospecific and reversible by (+)bicuculline, picrotoxin, and naloxone (Kavaliers and Wiebe, 1987). These findings suggest that the effect is mediated by both the GBRC and opioid systems. One of the major disadvantages responsible for the discontinuation of the clinical use of steroid anesthetics is the lack of analgesic effects (Holzbauer, 1976). Perhaps these recent observations will rekindle interest in identifying steroid anesthetics with analgesic properties.

Conclusion

By presenting some of the functional interactions between the known recognition sites on the GBRC, this review has attempted to provide some insight into the possible mechanism(s) and site(s) of action of the 3α-hydroxylated, 5α-reduced steroids. One feature is clear, namely, that the interactions of these neuroactive steroids with the GBRC are complex and their pharmacological effects may not all be mediated by a direct effect on the GBRC protein. One likely possibility is that some of these steroids interact with specific sites at the receptor protein–membrane lipid interface in a manner similar to the local anesthetics but, instead, to facilitate ion (i.e., Cl^-) conductance (Strichartz and Ritchie, 1985). The recent cloning of the cDNAs encoding the α and β subunits of the GBRC will allow the large scale production of the receptor complex by expression of the cDNAs. The expressed receptor–ionophore protein can be evaluated for the ability of the radiolabeled steroid to bind to the hydrophobic regions associated with α-helical domains lining the transmembrane portion of the channel. The presence of allosteric interactions with the other sites present can be further evaluated. The specificity of the effect may be determined by evaluating the ability of these steroids to interact with components of the receptor–ionophores with similar structural architecture (e.g., nicotinic acetylcholine and glycine receptors). Ultimately, these types of studies will be required to determine the site and mechanism of action of these neuroactive steroids.

The observations that steroids active at the GBRC have in vitro potencies greater than many clinically useful BZs, and that circulating plasma levels of 3α-OH-DHP, THDOC, and 5α-pregnanediol are sufficient to activate chloride conductance in the presence of GABA (Holzbauer, 1975), provide a compelling argument in favor of a physiological role for these steroids in the regulation of brain excitability. This provides the impetus not only to study the postsynaptic actions of these naturally occurring steroids but the regulation of their synthesis and degradation as well. The fluctuating levels of the active metabolites of progesterone during menses draw attention to the possible role of these metabolites in the etiology of PMS and catamenial epilepsy. Studies of this type may eventually provide rational treatment strategies for the mood disorders associated with reproductive cycles and pathological alterations in steroid levels. Nevetheless, it must be established whether these changes in levels are causal or epiphenomenon. Knowledge of the structure-activity requirements and site of action of the GBRC-active steroids will allow the design of anesthetics with greater specificity and fewer side effects. Perhaps specific antagonists can be designed so that the degree of anesthesia can be finely controlled. Similarly, partial agonists can be designed that would provide anxiolytic or anticonvulsant activity without sedative-hypnotic effects.

The existence of high affinity steroid modulation of the GBR ushers in a new phase in research on steroids with anxiolytic, anticonvulsant, and sedative-hypnotic properties. It

provides a focus on which the details of their mechanism(s) and site(s) of action can be defined. Taking advantage of the recent cloning of the GBRC and recombinant DNA technology, definitive experiments can be performed to establish the structure-function of this novel steroid recognition site(s). More important, it provides insight into another mechanism by which the endocrine system may influence brain function and behavior.

Acknowledgments

I am grateful to Roberta Brinton, Deborah Johnson, and Bruce McEwen for their discussions and critical review of this manuscript. The investigations performed in the author's laboratory were supported by a Pharmaceutical Manufacturer's Association Foundation (PMAF) Research Starter Grant. The author is also a recipient of a Faculty Development Award in Basic Pharmacology from the PMAF.

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