Published in final edited form as:

Psychoneuroendocrinology. 2009 December ; 34S1: S59–S66. doi:10.1016/j.psyneuen.2009.05.020.

The influence of the membrane on neurosteroid actions at GABA_A receptors

Gustav Akk¹, Douglas F. Covey², Alex S. Evers^{1,2}, Joe Henry Steinbach^{1,3}, Charles F. Zorumski^{3,4}, and Steven Mennerick^{3,4}

¹Department of Anesthesiology, Washington University School of Medicine 660 S. Euclid Ave. St. Louis, MO 63110

²Department of Developmental Biology, Washington University School of Medicine 660 S. Euclid Ave. St. Louis, MO 63110

³Department of Anatomy & Neurobiology, Washington University School of Medicine 660 S. Euclid Ave. St. Louis, MO 63110

⁴Department of Psychiatry, Washington University School of Medicine 660 S. Euclid Ave. St. Louis, MO 63110

Summary

Modern views of anesthetic neurosteroid interaction with the GABA_A receptor conceptualize steroid ligands interacting with a protein binding site on the receptor. It has generally been assumed that the steroid interaction/binding site is contained in an extracellular domain of the receptor, and that steroid interactions are of high potency, evidenced by the low aqueous ligand concentrations required to achieve potentiation of channel function. We have been considering implications of the observations that steroids are quite lipophilic and that recently identified putative steroid binding sites are in transmembrane domains of the receptor. Accordingly, we expect that both the effective plasma membrane steroid concentration and steroid pharmacophore properties will contribute to steady-state potency and to the lifetime of steroid actions following removal of free aqueous steroid. Here we review our recent studies that address the evidence that membrane partitioning and intracellular accumulation are non-specific contributors to the effects of anesthetic steroids at GABA_A receptors. We compare and contrast the profile of anesthetic steroids with that of sulfated steroids that negatively regulate GABA_A receptor function. These studies give rise to the view that the inherent affinity of anesthetic steroid for GABA_A receptors is very low; low effective aqueous concentrations are accounted for by lipid partitioning. This yields a very different picture of the interaction of neurosteroids with the GABA_A receptor than that of steroid interactions with classical intracellular steroid receptors, which exhibit inherently high affinity. These considerations have practical implications for actions of endogenous neurosteroids. Lipophilicity will tend to promote autocrine actions of neurosteroids at GABA_A receptors within cells that synthesize neurosteroids, and lipophilic retention will limit intercellular diffusion from the source of steroid synthesis. Lipophilicity

© 2009 Elsevier Ltd. All rights reserved

Corresponding author: Steven Mennerick Department of Psychiatry Washington University School of Medicine 660 S. Euclid Ave., Campus Box 8134 St. Louis, MO 63110 menneris@wustl.edu Phone: (314) 747-2988 Fax: (314) 747-2983.

Disclosures. The authors have no competing financial interests to disclose. The work described was funded by NIH grant GM47969.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and steroid access to the receptor binding sites also must be considerations in drug design if drugs are to effectively reach the target GABA_A receptor site.

Keywords

Anesthetic; GABA_A; lipophilic; neurosteroid; inhibition; excitability

Neuroactive steroids are powerful anesthetics (Gasior et al., 1999, Zorumski et al., 2000, Akk et al., 2007). From this perspective, the history of research into anesthetic drug actions has been demarcated into two historical phases. Originally, because of the strong correlation between anesthetic lipophilicity and behavioral anesthesia, it was believed that behavioral anesthesia results from a critical concentration of anesthetic accumulating (non-specifically) in membranes. There are contemporary extensions of this hypothesis invoking lipid interaction with ion channels (Cantor, 1998). Nevertheless, the second, modern phase of anesthetic research has been dominated by the realization that anesthetics also directly bind proteinaceous sites, and this binding mimics the stereoselectivity of anesthetic actions (Franks and Lieb, 1994, Eckenhoﬀ, 2001, Franks, 2008). In the case of neurosteroids and many other anesthetics, it is believed that neuronal GABA_A receptors are an important cellular substrate of anesthesia (as well as other important behavioral effects of neurosteroids and neuroactive steroids) (Belelli and Lambert, 2005, Akk et al., 2007, Franks, 2008). Therefore, modern ideas of anesthetic action are currently strongly influenced by the idea that specific binding to protein sites on GABA_A receptors account for many behavioral actions of steroids and other anesthetic drugs.

Recent work, including ours, suggests that for anesthetic neurosteroids, a model occupying a middle ground between these two historical poles is appropriate. Our past work suggests that steroid effects on GABA_A and GABA_C receptor function are strongly enantioselective (Hu et al., 1997, Li et al., 2006, Li et al., 2007a). Enantioselectivity is most often interpreted as evidence for a chiral, protein binding site. This is because ligand interaction with proteins involves a “handedness” preference given that proteins are made entirely of L- amino acids (Westover and Covey, 2004, Covey, 2008). Other recent molecular evidence from others suggests a specific binding site on the GABA_A receptor for neurosteroids (Hosie et al., 2006, Hosie et al., 2008). Also, there is ample evidence for GABA_A receptor subunit differences in steroid sensitivity (Mitchell et al., 2008). Taken together, these results are consistent with the modern view that a protein binding site is important for neurosteroid regulation of receptor function. On the other hand, evidence for a binding site from studies of mutated GABA_A receptors places the steroid binding site within transmembrane domains of the receptor (Hosie et al., 2006, Hosie et al., 2008). This suggests the possibility that steroids must partition into the membrane before they can access the protein binding site. Therefore, structural features of steroids that allow their partitioning into the plasma membrane may need to be accounted for when considering steroid properties like potency and time course of action.

A second class of neurosteroids non-competitively antagonizes GABA_A receptor function (Park-Chung et al., 1999, Eisenman et al., 2004). Our evidence suggests poor enantioselectivity and poor diastereoselectivity for the antagonistic actions of sulfated steroids. This raises the possibility that this class of neurosteroid action may not require a chiral protein binding site. We entertain the possibility that antagonist steroid actions may represent a direct effect on the membrane, which in turn affects receptor function in a relatively non-specific manner. Herein we review some of our recent work suggesting that non-specific features of both positive and negative neurosteroid modulation deserve consideration in drug design and in interpreting endogenous neurosteroid actions.

Potentiating steroids and effects of non-specific accumulation

Several recent, thorough reviews have focused on evidence for the specificity of steroid actions and factors related to the ligand-receptor interaction (Belelli and Lambert, 2005, Herd et al., 2007, Hosie et al., 2007). Here we focus on our recent work that has been motivated by the idea that no matter how good the “fit” between a ligand and its proteinaceous receptor site, steroid actions will be influenced by limited access to this site by membrane partitioning. If lipid solubility is essential to drug access, then overall potency of the drug will include contributions from both ligand-receptor affinity and non-specific lipophilicity (access). Here the concept of potency is treated empirically and is defined as the aqueous concentration of drug needed to achieve a certain fraction (usually 50%) of maximum effect. If partitioning into the plasma membrane is important for neurosteroid access to a transmembrane binding site, then potency, defined by aqueous concentration, may be somewhat misleading in important ways because the membrane concentration for a lipophilic steroid will be much higher than the aqueous concentration.

There is precedent for the idea that some drugs with transmembrane receptor targets may first require partitioning into the plasma membrane (Hille, 1977, Orser et al., 1997, Krasowski et al., 2001, Lee and MacKinnon, 2004, Suchyna et al., 2004, Hemmings et al., 2005). We developed tests of whether neuroactive steroids access their putative GABA_A receptor site by conventional aqueous access to an extracellular binding domain, or whether membrane diffusion might be required (Shu et al., 2004, Akk et al., 2005).

In one set of studies, we examined individual GABA-gated channel activity and compared receptors that were either exposed directly to aqueous steroid or were sealed off from direct access by a patch pipette. When steroid and GABA agonist are delivered directly to receptors from a cell-attached patch pipette, steroids modulate single-channel behavior in a stereotyped manner (Akk et al., 2005). However, steroid similarly modulates channel function even when bath applied and sealed from direct access to the receptors in the membrane beneath the recording pipette. This suggests that membrane partitioning and lateral diffusion is sufficient for steroid to reach its site on the receptor.

These results leave open the possibility that steroids may permeate the cell and access an intracellular site on the receptor. If, in contrast, lateral membrane diffusion governs steroid access to receptor sites, then excised membrane patches might allow us to isolate this mode of access. By excising membranes, we removed access to intracellularly accumulated steroid. We loaded neuroactive steroids into cells by bath application, then excised patches containing GABA_A receptors into a steroid-free bath solution. We found that steroid potentiation of channel activity persisted in these excised patches. Therefore, plasma-membrane diffusion alone suffices for steroid delivery to the receptor. Although intracellular access is not required, our results support a likely binding site toward the cytoplasmic side of the membrane. A steroid made membrane impermeant by the conjugation of an anionic Alexa Fluor moiety at carbon 17 is effective only when applied to the intracellular membrane face of excised patches (Akk et al., 2005).

Steroid gating and potentiation of GABA_A receptors have extremely slow deactivation kinetics. Once the cell has been exposed to steroid, washing the effect away is extremely slow (deactivation times of > 30 s for direct gating and for potentiation by steroids in cultured hippocampal neurons). For conventional ligand-receptor interactions, slow deactivation upon removal of free ligand is a hallmark of a high affinity ligand-receptor interaction (Colquhoun et al., 1977, Lester and Jahr, 1992). In the absence of free agonist driving a forward reaction, the liganded channel “prefers” to transition back and forth between open and closed-liganded states rather than to allow agonist dissociation and final channel closure. In the case of steroids,

however, an alternative explanation for slow steroid functional offset times is that the slow offset represents slow membrane partitioning rather than an inherently tight association (slow dissociation) of ligand from receptor. In this model, steroid repeatedly dissociates and reassociates from the membrane to receptor until finally the steroid partitions from the membrane. Estimated logP values (common logarithm of the octanol:water partition coefficient) of ~4 for neurosteroids (see below) make this explanation plausible.

To test this idea more directly, we reasoned that a lipophilic steroid scavenger could be used to distinguish these two alternative explanations for slow steroid offset/deactivation kinetics. Cyclodextrins represent such a scavenger (Adam et al., 2002, Bom et al., 2002). Cyclodextrins are membrane impermeant cyclic sugars with a hydrophobic core with which steroids and other lipophilic molecules can form an inclusion complex (Szejtli, 1998). Cyclodextrins interact with the plasma membrane, thus providing a hydrophobic binding pocket for sequestration of free membrane steroids as they diffuse within the membrane. In our hands, γ -cyclodextrin did not affect GABA_A receptor function at the concentrations we employed (Shu et al., 2004, Shu et al., 2007), but see (Pytel et al., 2006). Importantly, steroids bound to receptors will not be free to form an inclusion complex with γ -cyclodextrin. Therefore, if wash during the deactivation phase of steroid gating or potentiation is speeded by the presence of γ -cyclodextrin, this would be powerful evidence that membrane partitioning is rate limiting rather than steroid dissociation from a receptor site. In fact, we found that cyclodextrins dramatically speed washout of steroid direct gating and potentiation (Figure 1) (Shu et al., 2004, Shu et al., 2007), consistent with the idea that membrane partitioning rate-limits the steroid effects.

Fluorescent steroids to visualize accumulation

If non-specific accumulation effects dictate steroid actions at GABA_A receptors, it would be helpful to visualize this accumulation. We synthesized a fluorescent analogue of (3 α ,5 α)-3-hydroxypregnan-20-one (allopregnanolone or 3 α 5 α P) that retained activity at GABA_A receptors. We found that this steroid accumulated in lipophilic regions of the cell, including plasma membrane and in intracellular lipophilic compartments (Figure 2)(Akk et al., 2005). Imaging experiments that showed strong intracellular fluorescent steroid accumulation highlighted the possibility that although intracellular steroid is not necessary for receptor effects (see above), intracellular reservoirs in intact cells may influence steroid effects. We recorded steroid deactivation in the same cells in which we imaged the removal of intracellular steroid pools. The kinetics correlated well; loss of intracellular fluorescence corresponded to a slow component of current deactivation (Akk et al., 2005).

Interestingly, in addition to speeding the offset of steroid effects on GABA_A receptors, membrane-impermeant γ -cyclodextrin speeded the loss of intracellular fluorescence (Akk et al., 2005). Because cyclodextrin only sequesters plasma membrane steroid, this result indicates that plasma membrane and intracellular steroid pools are normally in equilibrium. By draining the plasma membrane pool with cyclodextrin, one observes the net loss of intracellular steroid to the plasma membrane. Unlike results with plain saline wash, in cells treated with cyclodextrin during the deactivation phase of steroid responses, the decay of electrical currents outpaced the loss of fluorescence (Akk et al., 2005). This indicates that equilibration of the intracellular reservoirs with the plasma membrane (the location of GABA_A receptors) is > ~9 s, which was the slow time constant of current deactivation during cyclodextrin wash (Akk et al., 2005). These imaging results complement the electrophysiology results and support the plausibility of membrane access to steroid receptor sites, with lipophilic intracellular compartments serving as physiologically important reservoirs.

One could imagine that under some conditions, intracellular steroid could serve as a steroid sink and slow the approach of steroid effects toward a steady state. This indeed seems to be

the case (Li et al., 2007b). The slow rise of steroid potentiation is absent in excised outside-out patches, suggesting that intracellular compartment reservoirs act as a sink, slowing the onset of steroid effects in whole-cell recordings. At low concentrations of steroid applied to whole cells, effects can take minutes to develop (Li et al., 2007b), a fact underappreciated in most electrophysiology experiments, where onset of ligand-activated currents is limited by aqueous diffusion and typically take < 1 s to develop, even at low ligand concentrations. Under these conditions we detected potentiation of GABA currents at $3\alpha 5\alpha$ P concentrations as low as 3 nM, a concentration likely present in the central nervous system.

There are several implications of the observations that steroid potency and life time of action is dictated partly by non-specific accumulation and lipophilicity. First, although steroids have a high apparent affinity for GABA_A receptors, the intrinsic affinity of steroid for the site is likely much lower. A logP value of 4.2 for $3\alpha 5\alpha$ P (see above) implies that for a given aqueous steroid concentration, plasma-membrane concentration, the concentration most directly relevant for steroid access to a site on the GABA_A receptor, will be 10,000-100,000 fold greater than the aqueous concentration. Thus, if the EC₅₀ of $3\alpha 5\alpha$ P is 100 nM for potentiation of GABA_A receptors ((Shu et al., 2004), the membrane concentration at this aqueous concentration will be near 1 mM. This value probably better reflects the true affinity of ligand for the receptor and suggests a very weak ligand-receptor interaction. This situation, therefore, is very different than the situation describing steroid actions on conventional intracellular steroid receptors. For instance, estradiol affinity for the estrogen receptor can be defined by classical binding studies to be < 0.1 nM (Escande et al., 2006). Because these assays are performed on recombinant, soluble receptor, the assays yield a value near the true k_d .

An implication of these considerations that is important for drug design is that nonspecific lipophilicity of steroids will influence potency and longevity of steroid actions at GABA_A receptors. This hypothesis can be tested using the two major naturally occurring neurosteroids. $3\alpha 5\alpha$ P (logP 4.2) and $3\alpha 5\alpha$ THDOC (logP 3.5), agents that differ in lipophilicity by a predicted 0.7 logP units (see Figure 3 for structures). Based on this difference, one would predict that ($3\alpha,5\alpha$)-3,21-dihydroxypregnan-20-one ($3\alpha 5\alpha$ THDOC) would exhibit a potency (aqueous concentration necessary for half maximum response) that is lower than $3\alpha 5\alpha$ P, and deactivation kinetics for $3\alpha 5\alpha$ THDOC should be faster. Our preliminary results support this hypothesis (Chisari et al., 2008). Notably, THDOC and $3\alpha 5\alpha$ P are well matched for pharmacophore properties, although one cannot be completely sure that the pharmacophore is completely unaltered. Both compounds contain a 3α -hydroxy substituent known to be essential for steroid activity at GABA_A receptors. Both also contain a methylketone substituent at carbon 17; a hydrogen bond acceptor at this position is also essential for high activity. Additional commercially available neuroactive steroids can be used to extend the range of logP values beyond those of the naturally occurring steroids. For instance, the intravenous anesthetic Althesin is comprised of alphaxalone and alphadolone, the 11-keto derivatives of $3\alpha 5\alpha$ P and $3\alpha 5\alpha$ THDOC respectively. The addition of this 11-keto group extends the range of logP values (without altering the above critical components of the pharmacophore) to 3.1 and 2.2 for alphaxalone and alphadolone respectively. Our preliminary experiments support the predictions of lower potency and faster deactivation kinetics with the less lipophilic steroids (Chisari et al., 2008).

With respect to naturally occurring steroids, the difference in lipophilicity could give rise to different physiological effects. Recently, it has been suggested that neurosteroid synthetic enzymes are localized within principal, projection neurons in many CNS regions, but not in interneurons (Agis-Balboa et al., 2006). For $3\alpha 5\alpha$ P, the more lipophilic steroid, autocrine actions would be expected to be particularly important because of lipophilic retention. Because steroidogenic enzymes are known to be regulated (Mellon and Griffin, 2002) this autocrine intracellular retention could conceivably have feedback effects on further steroid synthesis.

3 α 5 α THDOC, the more hydrophilic steroid, would more likely diffuse further from the cell of origin, possibly exerting paracrine influences.

Beyond simple logP values, different steroids may partition and diffuse differently within local membrane environments of different lipid composition (Alakoskela et al., 2007), giving rise to subtle cell type or even subcellular differences in steroid actions. To date, we have no direct evidence for such a source of heterogeneity in steroid actions under physiological conditions.

Studies of neurosteroid antagonists

Sulfated neurosteroids are fairly potent non-competitive (with respect to GABA and to potentiating steroids) antagonists of GABA_A receptor function (Park-Chung et al., 1999, Eisenman et al., 2003). It may be instructive to compare specificity of these compounds' actions with those of the more often studied anesthetic steroids. For this class of neurosteroids, the profile of effects (beyond the obvious negative versus positive modulation of receptor function) differs from that of anesthetic steroids. However, this profile, like that of anesthetic steroids, includes some evidence for specificity and some evidence of non-specificity. This latter class of result possibly implicates membrane interactions as a component of effects on GABA_A receptor function.

Several mutations in mammalian GABA_A receptor subunits eliminate sulfated steroid antagonism (Akk et al., 2001). Mutations have also been discovered in invertebrates that eliminate sulfated steroid effects on ionotropic GABA receptor function (Twede et al., 2007). In the case of the mammalian mutation, a single amino acid change near the cytoplasmic side of the α 1 TM2 domain (V256S) eliminates pregnenolone sulfate sensitivity (Akk et al., 2001). Because sulfated steroids are negatively charged and do not pass through the membrane efficiently (Eisenman et al., 2007), it is unlikely that this residue is involved in binding of sulfated steroids. Instead, this residue is likely critical to the transduction of steroid binding to inhibition of channel function, possibly an alteration of receptor desensitization (Shen et al., 2000). Evidence is stronger that multiple residues within the TM1 and TM2 domains of the invertebrate subunit participate in binding sulfated steroids (Twede et al., 2007), although it is difficult to exclude a transduction explanation completely. Therefore, specific residues that affect sulfated steroid sensitivity hint at, but do not prove, a specific binding site for this class of neurosteroid.

GABA_A receptor inhibition by neurosteroids exhibits fewer structural requirements than GABA_A receptor potentiation by anesthetic steroids. An anionic group (usually a sulfate group) at C3 is important, though not necessary for antagonism. 3 β -hydroxysteroids antagonize GABA_A receptors by a similar mechanism to anionic (sulfated steroids), albeit at lower potency. GABA-inhibiting steroids are nearly equally active with the charged substituent in the 3 α or the 3 β configuration and so exhibit no appreciable carbon 3 diastereoselectivity. Furthermore, steroid analogues with pregnene, 5 β - or 5 α -reduced pregnane, or a 5 β - or 5 α -reduced androstane structures can all support inhibition (Majewska et al., 1988, Majewska et al., 1990, Demirgoren et al., 1991, Park-Chung et al., 1999). Enantiomeric pairs for DHEAS, pregnenolone sulfate, and 3 α 5 β PS have been synthesized. In hippocampal neurons little enantioselectivity was found for pregnenolone sulfate or for 3 α 5 β PS, though significant enantioselectivity (~8 fold) was found for DHEAS (Nilsson et al., 1998). Therefore, on balance diastereoselectivity and enantioselectivity are weaker for sulfated steroid antagonism than for hydroxysteroid potentiation. These results hint at a non-specific component of steroid antagonism.

In contrast to potentiating steroid effects, the kinetics of offset for sulfated steroid antagonism are more rapid and exhibit no detectable cyclodextrin sensitivity (Shu et al., 2007). This negative result is not caused by an inability of cyclodextrins to form inclusion complexes with

sulfated steroids (Shu et al., 2007). Instead, this result could be consistent with the idea that sulfated steroid offset kinetics are dictated by conventional ligand-receptor dissociation kinetics. Alternatively, the results could suggest that sulfated steroid effects are mediated by steroid-membrane interactions that are inaccessible to cyclodextrin sequestration.

We recently found that sulfated steroids, but not potentiating hydroxysteroids, alter membrane capacitance (Mennerick et al., 2008). This effect differed from effects reported for other charged membrane probes. Classical literature suggests that many charged lipophilic molecules produce voltage-dependent capacitive charge movements as the change in applied transmembrane field causes a change in charge location across the membrane bilayer (Ketterer et al., 1971, Andersen and Fuchs, 1975, Bruner, 1975, Fernandez et al., 1983, Benz, 1988, Gonzalez and Tsien, 1995). By contrast, sulfated steroid capacitive currents exhibit no significant voltage dependence. This capacitance change exhibited a concentration-response not dissimilar to that for the effect of sulfated steroids on receptor function. There was a lack of diastereoselectivity and a lack of enantioselectivity. We suggest that this effect could represent a membrane interaction that is important to the antagonistic effects of sulfated steroids on GABA_A receptors. For instance, this could occur if membrane alterations, reflected in capacitance changes, allosterically alter channel behavior.

In preliminary experiments, we recently tested the idea that other structurally diverse amphiphiles with reported actions at GABA_A receptors (Sogaard et al., 2006) might act in a manner mechanistically similar to that of sulfated steroids. We found that detergents and other amphiphiles have complicated mixtures of potentiating and antagonizing actions at GABA_A receptors (Chisari et al., 2009). The antagonism is promoted by receptor activation and manifests as an apparent increase in desensitization. These features are superficially similar to sulfated steroid antagonism. Furthermore, amphiphile inhibition of channel function is abrogated by the V256S mutation, which eliminates sulfated steroid antagonism. By contrast the potentiating actions of the amphiphiles, which are revealed at low GABA concentrations, are unaffected by either the Q241L mutation in $\alpha 1$ subunits that eliminates steroid-mediated potentiation of GABA_A receptors or by the V256S mutation. Therefore, potentiating actions are distinguishable from the actions of anesthetic steroids while amphiphile negative regulation exhibits mechanistic similarity to sulfated steroid effects. Although this could involve direct binding to the receptor, it is also possible that membrane perturbations (e.g. membrane elasticity) by amphiphiles allosterically alter channel behavior (Sogaard et al., 2006). These preliminary results suggest that the mechanism of steroid antagonism extends to structurally diverse modulators and amplifies the possibility that membrane interactions could contribute to sulfated steroid antagonism of GABA_A receptor function.

In summary, sulfated steroids exhibit a pattern of functional effects and structure-activity profiles distinct from those of potentiating steroids. Arguing for selectivity of actions are mutations that abolish sulfated steroid antagonism. Furthermore, the offset time course of sulfated steroid effects, unlike the time course of potentiating steroid effects, is not sensitive to γ -cyclodextrin scavenging. This could be interpreted as evidence for a conventional binding site, rate limited by agonist dissociation rather than by residual membrane portioned steroid. Supporting non-selectivity are studies suggesting that the V256S mutation abolishes receptor sensitivity to structurally diverse amphiphilic compounds. Hinting at a role for non-specific membrane effects are studies showing a direct electrophysiological signature (membrane capacitance changes) of sulfated steroid interaction with membranes. A final argument for weak selectivity is the generally weak enantioselectivity exhibited by sulfated steroids.

Overall summary

In summary, our recent results argue for a more nuanced view of anesthetic neurosteroid interactions with the GABA_A receptor than either the outdated membrane fluidity hypotheses of anesthetic action or more conventional, modern views of proteinaceous binding sites. Our evidence suggests that anesthetic steroids must partition into the membrane before accessing a potentiating site on the receptor. In addition, intracellular accumulation can influence steroid actions by serving as a steroid reservoir. These nonspecific factors have important roles in dictating steroid potency and rate limit the lifetime of steroid actions following removal of free aqueous steroid. Furthermore, these non-specific requirements dictating steroid access need to be considered in drug design. There is also evidence that non-specific membrane interactions may participate in the antagonistic effect of sulfated steroids on GABA_A receptors, although the pattern of evidence is dissimilar from that of potentiating steroids, suggesting that membrane interactions play distinct roles in the two forms of modulation.

Acknowledgments

The authors thank their laboratory colleagues who contributed to the work herein.

References

- Adam JM, Bennett DJ, Bom A, Clark JK, Feilden H, Hutchinson EJ, Palin R, Prosser A, Rees DC, Rosair GM, Stevenson D, Tarver GJ, Zhang MQ. Cyclodextrin-derived host molecules as reversal agents for the neuromuscular blocker rocuronium bromide: synthesis and structure-activity relationships. *J. Med. Chem* 2002;45:1806–1816. [PubMed: 11960492]
- Agis-Balboa RC, Pinna G, Zhubi A, Maloku E, Veldic M, Costa E, Guidotti A. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A* 2006;103:14602–14607. [PubMed: 16984997]
- Akk G, Bracamontes J, Steinbach JH. Pregnenolone sulfate block of GABA_A receptors: mechanism and involvement of a residue in the M2 region of the α subunit. *J. Physiol. (Lond.)* 2001;532:673–684. [PubMed: 11313438]
- Akk G, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S. Mechanisms of neurosteroid interactions with GABA_A receptors. *Pharmacol. Ther* 2007;116:35–57. [PubMed: 17524487]
- Akk G, Shu HJ, Wang C, Steinbach JH, Zorumski CF, Covey DF, Mennerick S. Neurosteroid access to the GABA_A receptor. *J. Neurosci* 2005;25:11605–11613. [PubMed: 16354918]
- Alakoskela JM, Covey DF, Kinnunen PK. Lack of enantiomeric specificity in the effects of anesthetic steroids on lipid bilayers. *Biochim. Biophys. Acta* 2007;1768:131–145. [PubMed: 16945324]
- Andersen PS, Fuchs M. Potential energy barriers to ion transport within lipid bilayers. Studies with tetraphenylborate. *Biophys. J* 1975;15:795–830. [PubMed: 1148364]
- Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the GABA_A receptor. *Nat. Rev. Neurosci* 2005;6:565–575. [PubMed: 15959466]
- Benz R. Structural requirement for the rapid movement of charged molecules across membranes. Experiments with tetraphenylborate analogues. *Biophys. J* 1988;54:25–33. [PubMed: 3416031]
- Bom A, Bradley M, Cameron K, Clark JK, Van Egmond J, Feilden H, MacLean EJ, Muir AW, Palin R, Rees DC, Zhang MQ. A novel concept of reversing neuromuscular block: chemical encapsulation of rocuronium bromide by a cyclodextrin-based synthetic host. *Angew. Chem. Int. Ed. Engl* 2002;41:266–270. [PubMed: 12491405]
- Bruner LJ. The interaction of hydrophobic ions with lipid bilayer membranes. *J. Membr. Biol* 1975;22:125–141. [PubMed: 1170333]
- Cantor RS. The lateral pressure profile in membranes: a physical mechanism of general anesthesia. *Toxicol. Lett* 1998;100–101. 451–458.
- Chisari, M.; Bandyopadhyaya, AK.; Covey, DF.; Mennerick, S.; Zorumski, CF. 2008 Neuroscience Meeting Planner. Vol. vol. Program number 531.13. Society for Neuroscience; Washington, D.C.: 2008. Neurosteroid lipid solubility and GABA_A receptor potentiation.

- Chisari, M.; Shu, HJ.; Taylor, A.; Steinbach, JH.; Zorumski, CF.; Mennerick, S. GABA_A receptor modulation by neurosteroids and by structurally diverse amphiphiles. In: Panzica, GC.; Gotti, S., editors. 5th International Meeting: Steroids and the Nervous System. Trabajos Del Instituto Cajal; Torino, Italy: 2009. p. 190
- Colquhoun D, Large WA, Rang HP. An analysis of the action of a false transmitter at the neuromuscular junction. *J. Physiol. (Lond.)* 1977;266:361–395. [PubMed: 192885]
- Covey DF. ent-Steroids: Novel tools for studies of signaling pathways. *Steroids*. 2008
- Demirgoren S, Majewska MD, Spivak CE, London ED. Receptor binding and electrophysiological effects of dehydroepiandrosterone sulfate, an antagonist of the GABA_A receptor. *Neuroscience* 1991;45:127–135. [PubMed: 1661387]
- Eckenhoff RG. Promiscuous ligands and attractive cavities: how do the inhaled anesthetics work? *Mol. Interv* 2001;1:258–268. [PubMed: 14993365]
- Eisenman, LN.; He, Y.; Covey, DF.; Zorumski, CF.; Mennerick, S. Potentiation and inhibition of GABA_A receptor function by neuroactive steroids. In: Smith, SS., editor. *Neurosteroid Effects in the Central Nervous System*. CRC Press; Boca Raton: 2004. p. 95-117.
- Eisenman LN, He Y, Fields C, Zorumski CF, Mennerick S. Activation-dependent properties of pregnenolone sulfate inhibition of GABA_A receptor-mediated current. *J. Physiol. (Lond.)* 2003;550:679–691. [PubMed: 12938673]
- Eisenman LN, Shu HJ, Akk G, Wang C, Manion BD, Kress GJ, Evers AS, Steinbach JH, Covey DF, Zorumski CF, Mennerick S. Anticonvulsant and anesthetic effects of a fluorescent neurosteroid analog activated by visible light. *Nat. Neurosci* 2007;10:523–530. [PubMed: 17322875]
- Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, Nicolas JC, Cavailles V, Balaguer P. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. *Biochem. Pharmacol* 2006;71:1459–1469. [PubMed: 16554039]
- Fernandez JM, Taylor RE, Bezanilla F. Induced capacitance in the squid giant axon. Lipophilic ion displacement currents. *J. Gen. Physiol* 1983;82:331–346. [PubMed: 6631402]
- Franks NP. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. *Nat. Rev. Neurosci* 2008;9:370–386. [PubMed: 18425091]
- Franks NP, Lieb WR. Molecular and cellular mechanisms of general anaesthesia. *Nature* 1994;367:607–614. [PubMed: 7509043]
- Gasior M, Carter RB, Witkin JM. Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders. *Trends Pharmacol. Sci* 1999;20:107–112. [PubMed: 10203866]
- Gonzalez JE, Tsien RY. Voltage sensing by fluorescence resonance energy transfer in single cells. *Biophys. J* 1995;69:1272–1280. [PubMed: 8534797]
- Hemmings HC Jr. Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL. Emerging molecular mechanisms of general anesthetic action. *Trends Pharmacol. Sci* 2005;26:503–510. [PubMed: 16126282]
- Herd MB, Belelli D, Lambert JJ. Neurosteroid modulation of synaptic and extrasynaptic GABA_A receptors. *Pharmacol. Ther* 2007;116:20–34. [PubMed: 17531325]
- Hille B. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol* 1977;69:497–515. [PubMed: 300786]
- Hosie AM, Clarke L, da Silva H, Smart TG. Conserved site for neurosteroid modulation of GABA_A receptors. *Neuropharmacology* 2008;56:149–154. [PubMed: 18762201]
- Hosie AM, Wilkins ME, da Silva HMA, Smart TG. Endogenous neurosteroids regulate GABA_A receptors via two discrete transmembrane sites. *Nature* 2006;444:486–489. [PubMed: 17108970]
- Hosie AM, Wilkins ME, Smart TG. Neurosteroid binding sites on GABA_A receptors. *Pharmacol. Ther* 2007;116:7–19. [PubMed: 17560657]
- Hu Y, Wittmer LL, Kalkbrenner M, Evers AS, Zorumski CF, Covey DF. Neurosteroid analogues. Part 5. Enantiomers of neuroactive steroids and benz[e]indenes: total synthesis, electrophysiological effects on GABA_A receptor function and anesthetic actions in tadpoles. *J. Chem. Soc., Perkin Trans* 1997;1:3665–3671.
- Ketterer B, Neumcke B, Lauger P. Transport mechanism of hydrophobic anions through lipid bilayer membranes. *J. Membrane Biol* 1971;5:225–245.

- Krasowski MD, Nishikawa K, Nikolaeva N, Lin A, Harrison NL. Methionine 286 in transmembrane domain 3 of the GABAA receptor beta subunit controls a binding cavity for propofol and other alkylphenol general anesthetics. *Neuropharmacology* 2001;41:952–964. [PubMed: 11747900]
- Lee SY, MacKinnon R. A membrane-access mechanism of ion channel inhibition by voltage sensor toxins from spider venom. *Nature* 2004;430:232–235. [PubMed: 15241419]
- Lester RA, Jahr CE. NMDA channel behavior depends on agonist affinity. *J. Neurosci* 1992;12:635–643. [PubMed: 1346806]
- Li P, Bracamontes J, Katona BW, Covey DF, Steinbach JH, Akk G. Natural and enantiomeric etiocholanolone interact with distinct sites on the rat $\alpha 1\beta 2\gamma 2$ L GABAA receptor. *Mol. Pharmacol* 2007a;71:1582–1590. [PubMed: 17341652]
- Li P, Shu HJ, Wang C, Mennerick S, Zorumski CF, Covey DF, Steinbach JH, Akk G. Neurosteroid migration to intracellular compartments reduces steroid concentration in the membrane and diminishes GABA-A receptor potentiation. *J. Physiol. (Lond.)* 2007b;584:789–800. [PubMed: 17761771]
- Li W, Covey DF, Alakoskela JM, Kinnunen PK, Steinbach JH. Enantiomers of neuroactive steroids support a specific interaction with the GABA_C receptor as the mechanism of steroid action. *Mol. Pharmacol* 2006;69:1779–1782. [PubMed: 16527905]
- Majewska MD, Demirgoren S, Spivak CE, London ED. The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the GABAA receptor. *Brain Res* 1990;526:143–146. [PubMed: 1964106]
- Majewska MD, Mienville JM, Vicini S. Neurosteroid pregnenolone sulfate antagonizes electrophysiological responses to GABA in neurons. *Neurosci. Lett* 1988;90:279–284. [PubMed: 3138576]
- Mellon SH, Griffin LD. Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol. Metab* 2002;13:35–43. [PubMed: 11750861]
- Mennerick S, Lamberta M, Shu HJ, Hogins J, Wang C, Covey DF, Eisenman LN, Zorumski CF. Effects on membrane capacitance of steroids with antagonist properties at GABA_A receptors. *Biophys. J* 2008;95:176–185. [PubMed: 18339741]
- Lester RA, Jahr CE. NMDA channel behavior depends on agonist affinity. *J. Neurosci* 1992;12:635–643. [PubMed: 1346806]
- Li P, Bracamontes J, Katona BW, Covey DF, Steinbach JH, Akk G. Natural and enantiomeric etiocholanolone interact with distinct sites on the rat $\alpha 1\beta 2\gamma 2$ L GABAA receptor. *Mol. Pharmacol* 2007a;71:1582–1590. [PubMed: 17341652]
- Li P, Shu HJ, Wang C, Mennerick S, Zorumski CF, Covey DF, Steinbach JH, Akk G. Neurosteroid migration to intracellular compartments reduces steroid concentration in the membrane and diminishes GABA-A receptor potentiation. *J. Physiol. (Lond.)* 2007b;584:789–800. [PubMed: 17761771]
- Li W, Covey DF, Alakoskela JM, Kinnunen PK, Steinbach JH. Enantiomers of neuroactive steroids support a specific interaction with the GABA_C receptor as the mechanism of steroid action. *Mol. Pharmacol* 2006;69:1779–1782. [PubMed: 16527905]
- Majewska MD, Demirgoren S, Spivak CE, London ED. The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the GABAA receptor. *Brain Res* 1990;526:143–146. [PubMed: 1964106]
- Majewska MD, Mienville JM, Vicini S. Neurosteroid pregnenolone sulfate antagonizes electrophysiological responses to GABA in neurons. *Neurosci. Lett* 1988;90:279–284. [PubMed: 3138576]
- Mellon SH, Griffin LD. Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol. Metab* 2002;13:35–43. [PubMed: 11750861]
- Mennerick S, Lamberta M, Shu HJ, Hogins J, Wang C, Covey DF, Eisenman LN, Zorumski CF. Effects on membrane capacitance of steroids with antagonist properties at GABA_A receptors. *Biophys. J* 2008;95:176–185. [PubMed: 18339741]
- Mitchell EA, Herd MB, Gunn BG, Lambert JJ, Belelli D. Neurosteroid modulation of GABA_A receptors: molecular determinants and significance in health and disease. *Neurochem. Int* 2008;52:588–595. [PubMed: 18055067]

- Nilsson KR, Zorumski CF, Covey DF. Neurosteroid analogues. 6. The synthesis and GABA_A receptor pharmacology of enantiomers of dehydroepiandrosterone sulfate, pregnenolone sulfate, and (3 α , 5 β)-3-hydroxypregnan-20-one sulfate. *J Med Chem* 1998;41:2604–2613. [PubMed: 9651164]
- Orser BA, Pennefather PS, MacDonald JF. Multiple mechanisms of ketamine blockade of N-methyl-D-aspartate receptors. *Anesthesiology* 1997;86:903–917. [PubMed: 9105235]
- Park-Chung M, Malayev A, Purdy RH, Gibbs TT, Farb DH. Sulfated and unsulfated steroids modulate γ -aminobutyric acid_A receptor function through distinct sites. *Brain Res* 1999;830:72–87. [PubMed: 10350561]
- Pytel M, Mercik K, Mozrzymas JW. Interaction between cyclodextrin and neuronal membrane results in modulation of GABA_A receptor conformational transitions. *Br. J. Pharmacol* 2006;148:413–422. [PubMed: 16702996]
- Shen W, Mennerick S, Covey DF, Zorumski CF. Pregnenolone sulfate modulates inhibitory synaptic transmission by enhancing GABA_A receptor desensitization. *J. Neurosci* 2000;20:3571–3579. [PubMed: 10804198]
- Shu HJ, Eisenman LN, Jinadasa D, Covey DF, Zorumski CF, Mennerick S. Slow actions of neuroactive steroids at GABA_A receptors. *J. Neurosci* 2004;24:6667–6675. [PubMed: 15282269]
- Shu HJ, Zeng CM, Wang C, Covey DF, Zorumski CF, Mennerick S. Cyclodextrins sequester neuroactive steroids and differentiate mechanisms that rate limit steroid actions. *Br. J. Pharmacol* 2007;150:164–175. [PubMed: 17160009]
- Sogaard R, Werge TM, Bertelsen C, Lundbye C, Madsen KL, Nielsen CH, Lundbaek JA. GABA_A receptor function is regulated by lipid bilayer elasticity. *Biochemistry* 2006;45:13118–13129. [PubMed: 17059229]
- Suchyna TM, Tape SE, Koeppe RE 2nd, Andersen OS, Sachs F, Gottlieb PA. Bilayer-dependent inhibition of mechanosensitive channels by neuroactive peptide enantiomers. *Nature* 2004;430:235–240. [PubMed: 15241420]
- Szejtli J. Introduction and General Overview of Cyclodextrin Chemistry. *Chem Rev* 1998;98:1743–1754. [PubMed: 11848947]
- Twede VD, Covey DF, Tartaglia AL, Bamber BA. The neurosteroids dehydroepiandrosterone sulfate and pregnenolone sulfate inhibit the UNC-49 GABA receptor through a common set of residues. *Mol. Pharmacol* 2007;72:1322–1329. [PubMed: 17715402]
- Westover EJ, Covey DF. The enantiomer of cholesterol. *J Membr Biol* 2004;202:61–72. [PubMed: 15702370]
- Zorumski CF, Mennerick S, Isenberg KE, Covey DF. Potential clinical uses of neuroactive steroids. *Curr Opin Investigational Drugs* 2000;1:360–369.

Figure 1.

The effect of γ -cyclodextrin, a steroid scavenger, on the offset times of various steroid effects. These experiments were performed on cultured postnatal hippocampal neurons grown for 8-12 days in vitro. Whole-cell recordings were performed in the presence of glutamate receptor blockers and a pipette solution containing cesium methanesulfonate. Cells were clamped at -20 mV; thus chloride flow was inward. **A.** Effect of γ -cyclodextrin (500 μ M) on direct gating of GABA receptors (in the absence of GABA) by 300 nM 3 α 5 α P. Horizontal bars indicate time of application of the various compounds. **B.** Effect on the offset of GABA receptor potentiation upon removal of steroid and wash back to GABA alone. GABA concentration was 0.5 μ M. 3 α 5 α P concentration was 300 nM, and γ -cyclodextrin concentration was 500 μ M. **C.** Effect of

500 μM γ -cyclodextrin on antagonism offset (5 μM pregnenolone sulfate, PS). For this experiment, GABA concentration was 2 μM . The data in B and C are adapted from (Shu et al., 2007).

Figure 2.

Fluorescence images of a plasma membrane marker (DiI; left panel) and 100 nM of an NBD-tagged fluorescent steroid middle panel; (Akk et al., 2005) in hippocampal neurons. Note the strong intracellular accumulation. The right panel is a merged image. Scale bar is 10 μ .

Figure 3.

Various naturally occurring steroids (3α,5αP and 3α,5αTHDOC) and synthetic anesthetic analogues (alphaxalone and alphadolone). Estimated logP values are given from an average of 11 different algorithms (ALOGPS 2.1, <http://www.vcclab.org/lab/alogs/>; ChemAxon, <http://www.chemaxon.com/marvin/sketch/index.jsp>) demonstrating lipophilicity range of steroids with GABA_A receptor actions.