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Fishing for allosteric sites on $GABA_A$ receptors

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Abstract

$GABA_A$ receptors have structural and functional homology with a super-family of cys-loop ligand-gated ion channel receptors including the nicotinic acetylcholine receptors. Amino acid residues involved in ligand-binding pockets are homologous among super-family members, leading to the multiple-loop model of binding sites situated at subunit interfaces, validated by structural studies on the nicotinic acetylcholine receptor and water-soluble snail acetylcholine binding protein. This article will briefly review the literature on the agonist binding sites on the receptor super-family, and then describe the current situation for attempts to identify sites for allosteric modulators on the $GABA_A$ receptors. A combination of mutagenesis and photoaffinity labeling with anesthetic ligands has given some leads in this endeavor. Current work by others and ourselves focuses on three putative sites for modulators: (1) within the ion channel domain TM2, near the extracellular end; (2) the agonist binding sites and homologous pockets at other subunit interfaces of the pentameric receptor; and (3) on the linker region stretching from the agonist site loop C to the top of the TM1 region. It is likely that concrete structural information will be forthcoming soon.

1. Introduction

The $GABA_A$ receptors (GABAR) are ligand-gated chloride channels that mediate the bulk of rapid inhibitory synaptic transmission as well as tonic inhibition involving extrasynaptically localized GABAR [1,2]. GABAR have structural and functional homology with a super-family of cys-loop ligand-gated ion channel (LGIC) receptors including the nicotinic acetylcholine receptors (nAChR), glycine receptors, and 5HT3 receptors [3,4]. Amino acid residues involved in ligand-binding pockets are homologous among super-family members, leading to the multiple-loop model of binding sites situated at subunit interfaces [5]. This model has been validated by structures determined by X-ray crystallography on the pentameric water-soluble snail acetylcholine binding protein (AChBP) with homology to the extracellular domain of the nAChR, solved in the absence [6] and presence [7] of bound ligands.

GABAR are unique among neurotransmitter receptors in the number of allosteric ligands that modulate GABAR function. Fig. 1 shows the four general categories of sites that are present on this single protein, known for over 20 years. However, the allosteric site for anesthetic modulators involves numerous chemical classes that share more or less the same pharmacology, but might be expected to have unique binding sites.

GABAR are pharmacologically defined by a series of agonists, competitive and noncompetitive antagonists, and multiple types of allosteric modulators. The GABA site ligands include agonists muscimol, 3-aminopropane sulfonate, THIP, piperidine-4-sulfonate, and imidazole-acetic acid, and antagonists bicuculline and SR95531 = gabazine [9]. GABAR are inhibited noncompetitively by the plant convulsant picrotoxin [8,10] and synthetic cage convulsants, typified by *t*-butyl bicyclophosphorothionate [11]. The third site on the GABAR is the benzodiazepine (BZ) site that provides for modulation by this clinically important category of drug. The different isoforms of GABAR with differing subunit composition are particularly heterogeneous with respect to this site [12].

Finally, the allosteric modulator site was postulated to explain the pharmacologically specific direct interaction of barbiturates and related CNS depressant drugs with the GABAR protein, demonstrated by enhancement of both

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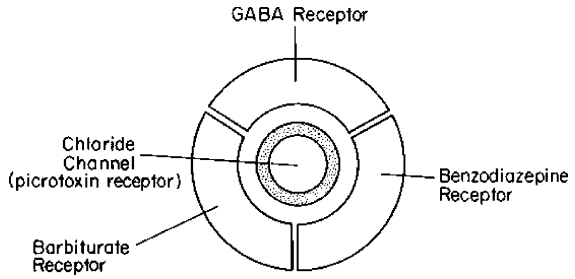

Fig. 1. $GABA_A$ receptor showing 4 different binding site domains (GABA, picrotoxin/convulsant, benzodiazepines, and anesthetic/allosteric modulators). Modified from [8].

function and in vitro radioligand-binding [8]. Because modulation is seen for ligands of diverse chemical structure (Fig. 2), including barbiturates, steroids, other intravenous anesthetics like etomidate, propofol, long-chain alcohols as well as ethanol, and also volatile anesthetics [10,13], multiple binding sites for modulatory ligands are likely rather than the schematic single site shown (Fig. 1).

2. Structure of ligand-gated ion channel receptor super-family members: identification of ligand-binding domains

The LGIC receptors of the cys-loop type, exemplified by the nAChR, are membrane-spanning pentamers, usually heteromeric, although the subunits are all structurally homologous to each other and contribute equally to the pseudo-symmetric structure that includes the ion channel as a central pore [4,5,14,15]. The amino acid residues contributing to the acetylcholine binding pocket, for agonists and antagonists, have been identified by a mixture of affinity labeling and site-directed mutagenesis approaches. It is very important to combine these two approaches because mutagenesis alone is subject to ambiguity in interpretation of results. Mutations can be tested for modified ligand-binding using functional assays and radioligand-binding assays, but changes in apparent agonist or antagonist affinity can result from mutations involving ion channel sites, that are allosterically coupled to agonist sites, or from other regions involved in conformational coupling to channel gating, or from regions involved in subunit-subunit interaction that are required for coupling of agonist binding to channel gating [5].

Thus several distinct domains were identified as points of contact for acetylcholine agonists or antagonists in nAChR (review [5]). The amino acid residues in the acetylcholine site could be shown to involve more than one subunit and produce a ligand pocket at the interface of subunits α–γ and α–δ [15,16]. The residues come from at least five distinct loops of sequence that come together in three-dimensional space, three on the 'major' subunit (α) and others on the 'minor' subunit (γ or δ) [17,18]. Homologous residues participate in ligand-binding in other LGIC receptor super-family members [5], including GABAR (Fig. 3 [4]). Interestingly, in GABAR the GABA binding site (two copies) occurs, like that for acetylcholine, at an interface between two subunits, β and α, involving residues to make the five homologous loops seen in nAChR. Surprisingly, the site for benzodiazepine (BZ) binding appears to be at the interface of α and γ subunits, involving amino acid residues homologous to those in the GABA site, i.e., the BZ site is a modified GABA site [4,5].

The biochemical studies were aided by using nAChR from the electric ray *Torpedo*, which contains the highest concentration of any receptor in nature. Receptor-rich

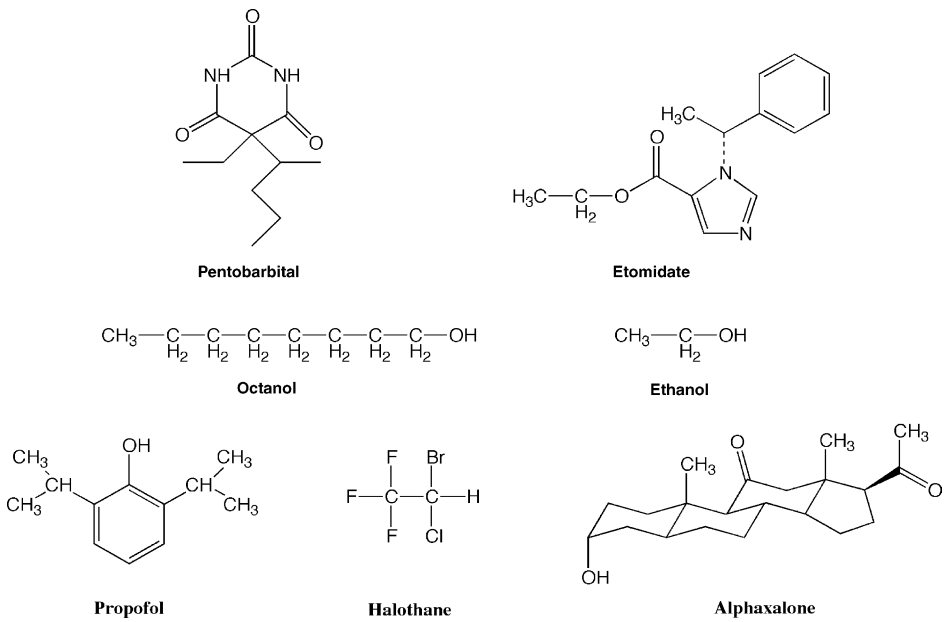

Fig. 2. Chemical structures of the GABAR allosteric modulators: pentobarbital, etomidate, *n*-octanol, ethanol, propofol, halothane, and neuroactive steroid (the anesthetic alphaxalone).

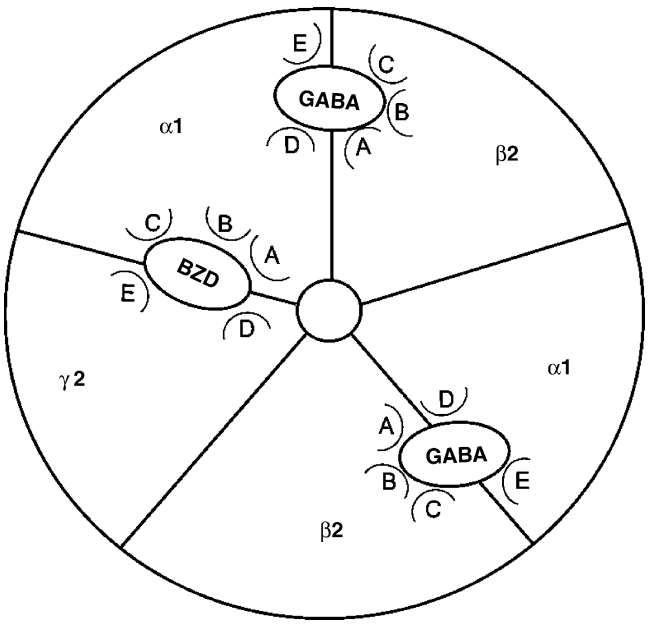

Fig. 3. GABAR 'Donut' model of heteropentameric protein with central pore and multiple-loop ligand-binding sites at subunit interfaces. GABA and BZ sites are indicated at β–α and α–γ subunit interfaces, respectively. Protein interaction loops A–E are indicated for each ligand site. Modified from [4]. The subunit arrnangement has been suggested by [27,72].

membranes with near-crystalline arrays of nAChR have been analyzed by computer-enhanced imaging with cryo-electron microscopy. These pictures yield a 4 Å resolution image of the pentamer, including the membrane-spanning domains that appear as rod-shapes believed to contain some α-helix structure. Ligand-binding domains can be identified in the extracellular portion, as well as a conformational change triggered by agonist binding (state-dependent structure) [14,15]. The extracellular half of the protein, including the acetylcholine binding sites can be compared to the structure of an acetylcholine binding protein, the snail AChBP, a homopentamer that binds acetylcholine and is homologous to the extracellular portion of nAChR [6]. This structure verified the identification of ligand-binding domains including the subunit interface and multiple loop concept [18], which was further supported by subsequent crystal structures of AChBP with bound agonist ligands, nicotine or carbamylcholine [7].

The GABAR is homologous with the nAChR. Homologous amino acid residues important for binding of ligands in nAChR were identified as participating in the GABA binding site at α–β interfaces (Fig. 3) as determined by mutagenesis [19]. As noted, homologous residues at the α–γ interface participate in the BZ binding pocket [20], while residues needed for functional allosteric coupling of BZ and agonist are outside the usual agonist pocket loops [21]. Three of the amino acids in the ligand-binding pocket loops were verified by this lab, using photoaffinity labeling with three different ligands, and microsequencing of affinity purified GABAR from bovine brain. Residue F64 (loop D in the minor subunit, α) was identified with the GABA ligand [^{3}H]muscimol [22]. Residue H101 (loop A in the major subunit, α) was identified with the BZ agonist [^{3}H]flunitrazepam by two independent labs [23,24]. Residue Y209 (loop C in the major subunit, α) was identified with the BZ partial inverse agonist [^{3}H]Rol5-4513 [25]. Although few in number so far, these affinity labeling studies support the conclusions on GABAR generated by the mutagenesis approach, as well as the homology to the super-family members for ligand-binding pocket loops. GABAR structure in the extracellular domain could be modeled with some approximations by threading the sequence onto the AChBP structure (e.g. [26,27]. This modeling can be used not only to examine residues of contact for ligand-binding [25,28], but also to postulate domain interactions and conformational changes associated with channel gating [29,30].

3. Modulator/anesthetic sites

Fig. 2 shows examples of some of the allosteric modulators of GABAR. All of these agents are active as general anesthetics, and enhancement of GABAR is the most compelling candidate mechanism for their anesthetic actions [8,10,13]. Attempts to identify the molecular sites on GABAR for the action of modulators have followed the classical approach of comparing the sequence of subunits that differ markedly in their sensitivity to a given ligand. Construction of chimeric subunits could narrow down the sequence of interest, and then point mutagenesis could be used to identify the important residue(s). Mutations are analyzed in recombinant expression systems such as *Xenopus* oocytes.

3.1. The mutagenesis approach: sequence scanning

3.1.1. Volatile anesthetics and high dose ethanol

In agreement with studies on organisms and on neurons, general anesthetics like isoflurane and ethanol (EtOH) enhance GABAR and glycine receptor channel function, whereas these drugs do not enhance the ρ subunits of GABAR [31]. Chimeric constructs with portions of ρ subunits swapped with non-ρ (α, β, γ) showed that the differential sensitivity is due to the domain encompassing membrane-spanning regions M1, M2, and M3, and the intervening linker sequences. This led to the identification of two residues in TM2 and TM3 in GABAR and glycine receptors that are required for anesthetic sensitivity. Replacing the residues in α2 with the residues from ρ (S270I, A291W) leads to loss of anesthetic enhancement [32].

Several additional lines of evidence support the idea that these two residues are part of an anesthetic binding pocket. First of all, they are located at the very 'top' of the membrane-spanning domains, at the extracellular side of the membrane lipids, and near to each other. They are not within the lumen of the pore, but situated behind the alpha

helices that form the ion channel. Sufficient room is apparently present at this position for a ligand-binding site, based on homology models based on crystal structures of the AChBP and the mechanosensitive receptor MSL [31]. A similar cavity was observed in the 4 Å resolution cryo-electron micrograph of nAChR [14]. The effect does depend on the subunit composition of the GABAR pentamer, notably, mutations in α and β have smaller effects on anesthetic sensitivity in certain αβ receptors lacking the γ2 subunit [33]. Mutation at these TM2 and TM3 sites affects the opening probability of the receptor and shifts the apparent affinity for GABA to the left. Examining a series of mutations at these sites showed that the modulation of GABA currents by anesthetics is dependent on the volume of the amino acid. This is consistent with a finite volume for ligand occupation; further, the size of amino acid at this position affects volatile general anesthetics differentially depending on the size and shape of the anesthetic molecule [34]. The volume of residue at these positions also affects the cut-off phenomenon, in which anesthetic potency of long-chain alcohols increases with carbon #, up to a point (cut-off) where activity no longer increases or is abruptly totally lost, consistent with a finite size of binding pocket. Larger substitutions at the critical residues lowers the cut-off size for alcohols, consistent with a binding pocket [35]. Finally, mutation of these residues by a cysteine residue led to the development of sensitivity to irreversible activation by an anesthetic molecule derivatized with a sulfhydryl reagent, i.e., an affinity label. Thus, an anesthetic molecule able to bind covalently to a cysteine residue shows irreversible modulation of function only when a cysteine is mutated into the receptor at the putative anesthetic binding site [36]. Taken together, these observations support the idea that an anesthetic binding pocket is positioned at the membrane/extracellular interface of the TM2 and TM3 helices [31,32].

3.1.2. Intravenous anesthetics

The mutations at the extracellular end of TM2 and TM3 that affected volatile anesthetic sensitivity were reported to not affect the sensitivity to intravenous anesthetics [37], e.g., the modulation by steroids appeared to be affected by the domain on the N-terminal side of TM2 in α2 [38], examining the same chimerae mentioned in [32]. Nevertheless, mutation in the TM3 of β subunits affects sensitivity to barbiturates [39], and chimerae of β3 with ρ suggest involvement of the membrane-spanning domains M1/2/3 for barbiturate modulation [40]. Further, the same residue identified by Mihic et al. [32] in TM2 of α subunits for volatile anesthetic sensitivity was already known to affect differential sensitivity of β subunits to barbiturates [41] and etomidate [42,43]. In fact, although the total knockout mouse for the GABAR β3 subunit had only modestly reduced sensitivity to anesthetics [44], the point mutation β3 N256M in TM2 "knocked-in" to a mouse totally eliminated the anesthetic action of etomidate [12]. This provides additional evidence for residues at the extracellular end of TM2 involved in anesthetic modulation, possibly involving a modulator binding site.

A third membrane-spanning helix amino acid, this one at the extracellular end of TM1, was identified by this lab using ρ versus non-ρ sequence scanning [45]. Modulation of GABAR binding by pentobarbital was shown to involve the N-terminal half of the α/β/γ subunits prior to the TM1 and TM2 domains. Fig. 4 shows the GABAR subunit sequence alignments in this region. A single amino acid β2G219 was identified that, when mutated to F, the amino acid found in ρ, eliminated modulation of binding and enhancement of GABAR currents by not only barbiturates, but also by the steroid anesthetic alphaxalone, etomidate, and propofol. Mutation of the homologous residue in α1 had an effect although smaller [45]. This mutation increased opening probability, and shifted the apparent affinity for GABA to the left. Also, direct gating by pentobarbital was not lost but the apparent affinity shifted to the left, and the desensitization rate to both GABA and direct activators was reduced [46]. A further study on a series of amino acid replacements at this position revealed that the extent of the left-shift for GABA as well as the extent of the reduction in enhancement of GABA by the

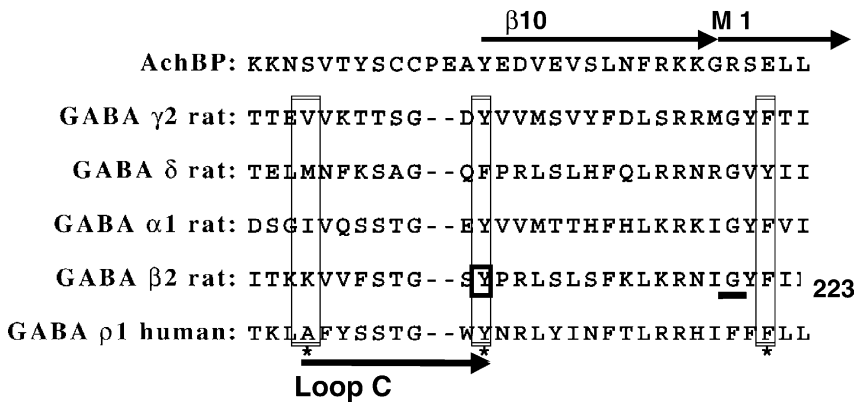

Fig. 4. Sequence alignment of GABAR subunits and AChBP for the Pre-TM1 region, from loop C of the GABA binding site, through β10, to TM1. The comparison of anesthetic-insensitive ρ subunit with α, β, γ, and δ subunits led to our discovery of the importance of TM1 residue β2G219. Also indicated (*) are some of the homologous amino acids identified in nAChR by photolabeling with anesthetic ligands by Cohen and collaborators.

four chemical classes of anesthetics were sensitive to the volume of amino acid in this position [47]. This indicated a 'pocket' at this position whose volume affected the extent of anesthetic modulation, either by allosteric coupling, or via a binding pocket for the ligands. Using the AChBP modeling approach, this residue in TM1 appeared to be positioned several Å away from the ''Mihic residues'' in TM2 and TM3 and further out of the membrane lipids (not shown), so probably not part of the same pocket [31]. On the other hand, the TM1 residue β2 G219 is near the portion of TM1 that apparently contacts TM2 and thus is positioned to modulate channel gating according to the proposed structure of Unwin and colleagues [14].

3.1.3. Low dose EtOH and maybe other drugs

A recent development suggests that the likely in vivo targets for EtOH are the GABAR subtypes that contain the δ subunit [48] and are positioned extrasynaptically, where they mediate responses to synaptic spill-over and/or tonic inhibition in response to ambient levels of GABA, thought to be in the 0.3–1 μM concentration range [2]. This lab found that the δ subunit was more sensitive to EtOH than the γ2 by about three-fold, and that, unexpectedly, the β3 subunit was more sensitive than β2 and β1 [49] to EtOH enhancement of GABA [48]. The δ subunit is associated exclusively with the α6 subunit in cerebellum and the α4 subunit in forebrain [50] and the α4β3δ and α6β3δ are the GABAR isoforms most sensitive to EtOH [48]. The α4β3δ combination in forebrain might well mediate alcohol effects on such functions as sleep (thalamocortical circuits), anxiety (limbic system), memory (hippocampus), and cognition (frontal cortex).

It is feasible that extrasynaptic GABAR, mediating tonic inhibition, possibly with different subunit composition in different cells [51,52], are physiologically most important in modulation by anesthetics, and thus a good 'fishing hole' in which to search for protein sites of alcohol and anesthetic action. These δ subunit-containing GABAR appear to be the most sensitive to not only EtOH [48] but also to other modulatory anesthetics and neuroactive steroids [52–54]. We propose [48,55] that δ-containing GABAR have a low efficacy for GABA and that this efficacy can be increased dramatically by low pharmacologically relevant concentrations of intravenous anesthetics such as etomidate and THDOC. Thus extrasynaptic receptors have potential to show increased activity by allosteric modulators, a concept gaining popularity [56]. Increased activity of extrasynaptic GABAR by anesthetics and EtOH is expected to lead to an overall dampening of neuronal excitability in neurons expressing GABAR δ subunits.

Is there a high affinity EtOH binding site on this GABAR subtype? Could these GABAR be the target of EtOH in causing effects on the brain? The subunit selectivity for EtOH surprisingly appears to be even more selective than that for other ligands with more complicated structures and binding elements. Support for the idea of a specific site might be provided by studies aimed at determining if single amino acid substitutions in these subunits (α4/6β3δ) change EtOH sensitivity markedly. A naturally occurring allele of α6, R100Q, was reported to occur in a rat strain selected for hypersensitivity to the motor in-coordinating effects of EtOH, the alcohol non-tolerant (ANT) rat. This allele appeared to correlate with greater behavioral sensitivity to benzodiazepines in these animals, but could not be related to the supersensitivity to EtOH [57]. The wild type α6(100R) expressed recombinantly is insensitive to agonist BZ such as diazepam when expressed with β and γ subunits, as opposed to the diazepam-sensitive GABAR α subunits (α1, 2, 3, 5) which have H at this position [58]. The mutated α6 with 100Q is partially sensitive to diazepam. None of the α subunits including the mutant expressed with β and γ2 were sensitive to EtOH [57].

We tested the allelic α6(R100Q) with β3 and δ in oocytes and found it to be even more sensitive than wild type α6 [55,59] to EtOH enhancement of GABA. Further, we found that the α6 allele 100Q was quite common in the Sprague–Dawley rat population. We are in the process of studying the pharmacology of this allele in vitro, and comparing rats with α6100Q/Q with wild type α6100R/R for EtOH sensitivity in behavior and cerebellar physiology.

This α6 residue 100 is part of the BZ site when expressed with γ2, and even the site of affinity labeling by flunitrazepam, in the homologous case of H101 in α1 [23,24]. It is also the residue that confers BZ-insensitivity on α4 and α6 subunits, and is the site of mouse knock-in strategy to dissect the subunit specificity of BZ action in vivo [12]. We are investigating whether this residue can affect EtOH sensitivity. In the EtOH-sensitive α6β3δ isoform, it appears that specific sequences of all three subunits are important to allosteric modulation. The possible role of 'GABA/BZ sites' like α6100 can be considered in combination with other lines of evidence (see below) that modulators may act on the agonist sites (presumably as co-agonists), or at the homologous 'agonist' domains on other subunit interfaces that do not bind the agonist for channel gating. Fig. 5 shows a model of the pentameric α6β3δ GABAR isoform, highlighting the potential sites of EtOH modulation at the agonist sites, two for GABA plus the other three. We are testing mutations including chimerae to examine domains responsible for the differences in α4/6 versus other α, β3 versus other β, and δ and γ2 with respect to EtOH sensitivity. In this manner we hope to identify structural elements involved in low dose EtOH (3–30 mM) modulation, relevant to in vivo EtOH actions and possibly therapeutics of alcoholism.

3.2. The photo-affinity labeling approach for anesthetic sites

Affinity labeling usually is specific for residues in or near the binding pocket for the ligand. Depending on the photochemistry, ligands can sometime diffuse some dis-

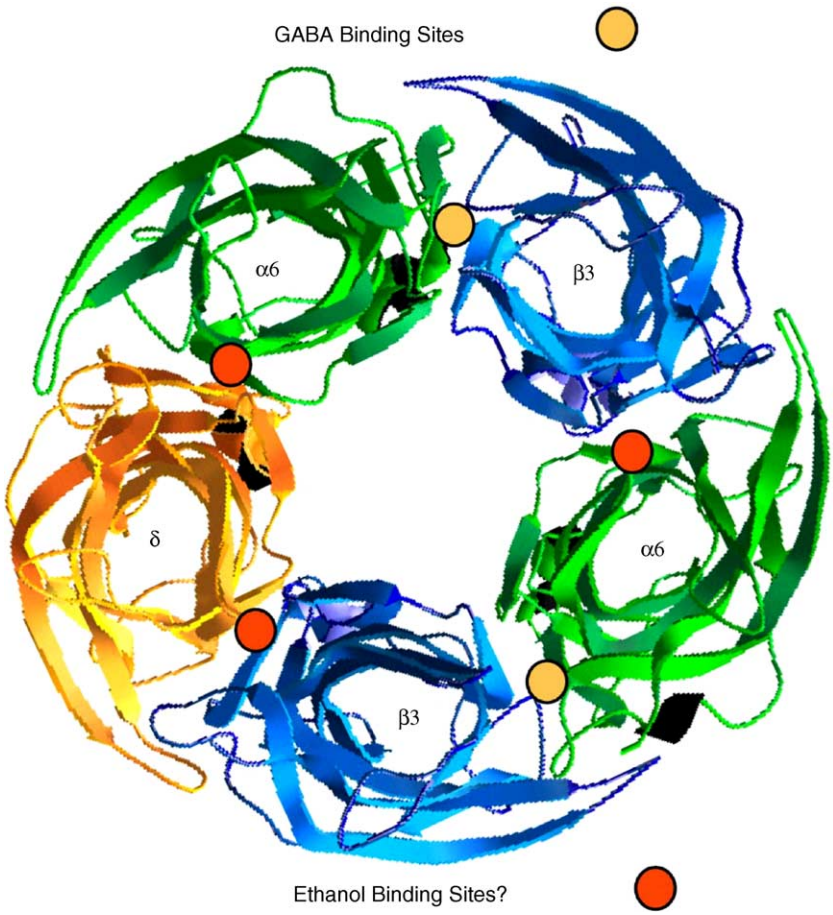

Fig. 5. 3D model of heteromeric α6β3δ GABAR showing potential ethanol sites corresponding to GABA sites or homologous regions on other subunits.

tance from the binding pocket before reacting with something (the target protein, other proteins, or solvent). Binding to non-target molecules, especially in crude tissue homogenates, is obviously the main drawback in affinity labeling. Nonspecific labeling can be minimized by using a high affinity ligand and tissue with a high concentration of sites to be labeled. This situation has been applied to good advantage with nAChR from *Torpedo* (e.g., the work of Cohen and colleagues), but unfortunately, this is not the case for anesthetic ligands, which are generally low potency, and GABAR in brain or recombinant expression cell systems, since it is a minor protein. Whatever the receptor concentration or ligand affinity, specificity can be supported by showing that photoincorporation of the labeled affinity ligand is protected against by inclusion of excess cold ligand for the site. In the case of allosteric modulators, one can also show receptor specificity by enhancement or inhibition of photolabeling by agonist, e.g., one might expect binding of anesthetic to be enhanced by GABA.

Thus the volatile anesthetic halothane, labeled with ^{14}C, without any chemical modification to introduce a photoreactive group, on the grounds that any modification would destroy anesthetic activity, has been used to look for photoaffinity labeling of brain proteins. Numerous proteins were labeled, most if not all of which are probably not the anesthetic receptor; proteins which are more abundant but not the real receptor may be labeled to a similar or greater extent than the target protein, possibly due to some structural features that make them relatively more sensitive than others, i.e., relative specificity [60].

However, this approach succeeded in identifying specific labeling in the *Torpedo* nAChR protein. Halothane was incorporated into several tyrosine residues in nAChR, but not into all accessible tyrosines [61]. Interestingly, incorporation was observed into three areas: γY105/Y111 but not Y104 (loop E of the agonist site), and this was inhibited by carbamylcholine; the second was δY212 (loop C of an interface homologous to the agonist binding site, and inhibited by curare but not agonist): this provides a suggestion that the agonist sites on homologous subunits might respond not only to BZ but to anesthetic modulators. The third was δY228 (TM1), but not Y218 [pre-TM1]. This last one was enhanced by agonist, and inhibited by cold isoflurane, evidence for pharmacological relevance. The δY228 in TM1 is two residues downstream from the homologous residue in TM1 of GABAR that we found to affect anesthetic modulation (β2G219 [47]). These amino acids are identified in Fig. 4.

The most potent and possibly most specific modulatory site on GABAR other than the BZ site is the neuroactive

Fig. 6. 3D model of GABAR β subunit showing GABA site, loop C (Y205 in β2), Pre-TM1 linker region (β10, and connection to TM1 at G219 (in β2). The pink color stands for α-helices and the white strip with an arrow at the end stands for β sheets. The green and red lines represent β9 and β10, respectively, based on the structure of snail AChBP [6]. Gly219 is located at the entrance to M1, and Tyr 205 (yellow) is one of the residues in loop C of the 'GABA binding site'. This area from loop C Y205 to TM1 G219 is suggested to mediate allosteric modulation by anesthetics.

steroid site. The target of action of the steroid anesthetic alphaxalone is generally accepted to be GABAR [10,31,62]. Neurosteroids are endogenous steroids believed to modify brain function via a non-genomic mechanism involving direct and rapid membrane action, primarily at GABAR [62]. As mentioned above the extrasynaptic δ subunit-containing GABAR are especially sensitive to steroids [52]. A radioactive, photo-incorporatable GABA-active steroid was synthesized and shown to photolabel proteins in brain. The major protein labeled was identified by the Evers group as a voltage-dependent anion channel, primarily of mitochondrial localization [63]. At first suspected to possibly have some role in modulating GABAR function, this protein was convincingly demonstrated to not play such a role and thus is considered not to contribute to anesthetic action [64]. However, this approach of using a high affinity, relatively specific anesthetic affinity label may yet prove successful.

Our team of anesthetic researchers, headed by Keith Miller at Massachusetts General Hospital in Boston, also has taken the approach of synthesizing radiolabeled anesthetic affinity labels. We have synthesized an azido derivative of octanol, 3-azi-octanol, which has anesthetic activity and modulates GABAR binding and function at 10–1000 μM and inhibits nAChR function at similar concentrations [65]. Labeled with [^{3}H], this ligand showed receptor-specific incorporation into the α subunit of *Torpedo* nAChR [66]. The primary site of incorporation was at αE262, near the extracellular end of TM2, and this was enhanced by acetylcholine. This amino acid is several residues away from the one identified in GABAR by Mihic et al. [32]. Lower level incorporation was found at the agonist binding site αY190, αY198 (both loop C), but not Y93; this was inhibited by excess carbamylcholine. A third site labeled without sensitivity to acetylcholine was in the TM4 area (H408 and S412), considered to be the protein-

lipid interface [66]. Thus the nAChR labeling by a long-chain alcohol anesthetic is consistent with evidence presented above for a site of action within the ion channel, near the extracellular membrane–water interface, with secondary consideration of the agonist site(s).

Of intermediate affinity is the anesthetic etomidate, which shows stereospecific enhancement of GABAR binding and function in brain slices at ca. 10 μM [8]. Modeling of steady-state data obtained by patch-clamp recording suggests that the binding affinity for etomidate to GABAR in the presence of GABA might be in the 1 μM ballpark [67]. We made an azido derivative of etomidate and showed it to have anesthetic activity and to enhance GABAR binding and function and to inhibit nAChR function at similar concentrations [68]. This molecule [^{3}H]azi-etomidate also photoincorporated into *Torpedo* nAChR α and δ subunits [69]. Highest degree and affinity of incorporation again involved the ion channel TM2, labeling αE262 and δQ276 near the extracellular end, and, lesser labeling, at δS258 and δS262 further in, all enhanced by acetylcholine. Secondly, incorporation was found in the agonist site, αY93 (loopA), αY190 and αY198 (loop C), and δD59 (loop D) but not γE57. Labeling of these sites is inhibited by agonist and modulated by other receptor ligands. Thus, again, an anesthetic binding to both the ion channel and the agonist site have been demonstrated by affinity labeling [69]. It is not known yet if these allosteric modulatory sites on nAChR, e.g., for halothane, octanol, and etomidate, will correspond to those responsible for anesthetic enhancement of GABAR.

We are using both of these tritiated photoaffinity label anesthetic ligands on GABAR purified from bovine brain [25] to attempt to identify binding site amino acids. We have obtained photoincorporation of [^{3}H]3-azi-octanol into brain GABAR observed on SDS–PAGE [70], as well as photoincorporation of [^{3}H]azi-etomidate into GABAR on SDS–PAGE in a band co-migrating with β subunit [71]. We are currently attempting to sequence these peptides to identify sites of attachment.

4. Conclusions and perspectives

Evidence to date suggests that anesthetic modulators of GABAR bind directly to the protein and that certain domains are most likely for points of contact. These include the ion channel TM2, especially the extracellular end, with possible contributions from similar domains of TM3 and TM1. Secondly, the agonist binding sites, as well as the homologous sites on other subunits in the pentamer, are implicated. We suggest that the ‘‘agonist binding sites’’ are very important and provide previously under-appreciated mechanistic insights. The coupling of agonist site binding to channel gating is beginning to be understood, just as we attempt to unravel the molecular action of allosteric modulators.

A third site of interest is the stretch of sequence we call pre-TM1: this spans loop C of the agonist site (or homologous domain of other subunits), down the beta sheet β10 (nomenclature of the AChBP), to the top of TM1. Fig. 4 shows the amino acids in GABAR subunits in the agonist binding site loop C, the residue G219 in β2 needed for intravenous anesthetic modulation, and the three areas labeled in nAChR by anesthetic affinity labels. Fig. 6 shows a three-dimensional model of this region. We suggest that binding of an anesthetic molecule somewhere in this domain would be able to leverage via β10 a conformational change in TM1, which is either part of the ion channel, or positioned near TM2, to affect ion channel gating. This model can be tested by suitable mutagenesis in the region and appropriate functional analysis.

Acknowledgments

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References

[1] Martin DL, Olsen RW, editors. GABA in the nervous system: the view at 50 years. Philadelphia: Lippincott Williams & Wilkins; 2000.
[2] Mody I. Distinguishing between $GABA_A$ receptors responsible for tonic and phasic conductances. Neurochem Res 2001;26:907–13.
[3] Olsen RW, Macdonald RL. $GABA_A$ receptor complex: structure and function. In: Egeberg J, Schousboe A, Krogsgaard-Larsen P, editors. Glutamate and GABA receptors and transporters: structure, function and pharmacology. London: Taylor & Francis; 2002. p. 202–19.
[4] Olsen RW, Sawyer GW. $GABA_A$ receptors. In: Lennarz W, Lane MD, editors. Encyclopedia of biological chemistry. San Diego: Elsevier; 2004 [in press].
[5] Galzi JL, Changeux JP. Ligand-gated ion channels as unconventional allosteric proteins. Curr Opin Struct Biol 1994;4:554–65.
[6] Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van der Oost J, Smit AB, et al. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 2001;411:269–76.
[7] Celie PHN. van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB, Sixma TK. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. Neuron 2004;41:907–14.
[8] Olsen RW, Fischer JB, Dunwiddie TV. Barbiturate enhancement of GABA receptor binding and function as a mechanism of anesthesia. In: Roth S, Miller K, editors. Molecular and cellular mechanisms of anesthetics. New York: Plenum Press; 1986. p. 165–77.
[9] Krogsgaard-Larsen P, Frolund B, Kristiansen U, Ebert B. Ligands for the $GABA_A$ receptor complex. In: Egeberg J, Schousboe A, Krogsgaard-Larsen P, editors. Glutamate and GABA receptors and transporters: structure, function and pharmacology. London: Taylor & Francis; 2002. p. 236–74.
[10] Olsen RW, Sapp DW, Bureau MH, Turner DM, Kokka N. Allosteric actions of CNS depressants including anesthetics on subtypes of the inhibitory $GABA_A$ receptor-chloride channel complex. In: Rubin E, Miller KW, Roth SH, editors. Molecular and cellular mechanisms of alcohol and anesthetics. Ann N Y Acad Sci 1991;625:145–54.

[11] Casida JE, Palmer CJ. 2,6,7-Trioxabicyclo[2.2.2]octanes: chemistry, toxicology and action at the GABA-gated chloride channel. In: Biggio G, Costa E, editors. Chloride channels and their modulation by neurotransmitters and drugs. New York: Raven Press; 1988. p. 109–23.
[12] Rudolph U, Mohler H. Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Ann Rev Pharmacol Toxicol 2004;44:475–98.
[13] Narahashi T, Arakawa O, Nakahiro M, Twombly DA. Effects of alcohols on ion channels of cultured neurons. In: Rubin E, Miller KW, Roth SH, editors. Molecular and cellular mechanisms of alcohol and anesthetics. Ann N Y Acad Sci 1991;625:26–36.
[14] Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. Nature 2003;423:949–58.
[15] Karlin A. Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci 2002;3:102–14.
[16] Pedersen SE, Cohen JB. *d*-Tubocurarine binding sites are located at α–γ and α–δ sub unit interfaces of the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 1990;87:2785–9.
[17] Chiara DC, Xie Y, Cohen JB. Structure of the agonist-binding sites of the *Torpedo* nicotinic acetylcholine receptor: affinity-labeling and mutational analyses identify γTyr-111/δArg-113 as antagonist affinity determinants. Biochemistry 1999;38:6689–98.
[18] Xie Y, Cohen JB. Contributions of *Torpedo* nicotinic acetylcholine receptor γTrp-55 and δTrp-57 to agonist and competitive antagonist function. J Biol Chem 2001;276:2417–26.
[19] Chang Y, Weiss DS. Functional domains of GABA receptors. In: Martin DL, Olsen RW, editors. GABA in the nervous system: the view at fifty years. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 127–39.
[20] Sigel E. Mapping of the benzodiazepine recognition site on $GABA_A$ receptors. Curr Top Med Chem 2002;2:833–40.
[21] Boileau AJ, Czajkowski C. Identification of transduction elements for benzodiazepine modulation of the $GABA_A$ receptor: three residues are required for allosteric coupling. J Neurosci 1999;19: 10213–20.
[22] Smith GB, Olsen RW. Identification of a [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric $acid_A$ receptor α subunit. J Biol Chem 1994;269:20380–7.
[23] Duncalfe LL, Carpenter MR, Smillie LB, Martin IL, Dunn SMJ. The major site of photoaffinity labeling of the $GABA_A$ receptor by [^{3}H]flunitrazepam is histidine 102 of the α subunit. J Biol Chem 1996;271:9209–14.
[24] Smith GB, Olsen RW. Deduction of amino acid residues in the $GABA_A$ receptor α subunits photoaffinity labeled with the benzodiazepine flunitrazepam. Neuropharmacology 2000;39:55–64.
[25] Sawyer GW, Chiara DC, Olsen RW, Cohen JB. Identification of the bovine γ-aminobutyric acid type A receptor α subunit residues photolabeled by the imidazobenzodiazepine [^{3}H]Ro15–4513. J Biol Chem 2002;277:50036–45.
[26] Cromer R, Morton CH, Parker MW. Anxiety over $GABA_A$ receptor structure relieved by AChBP. Trends Biochem Sci 2002;27:280–7.
[27] Ernst M, Brauchart D, Boresch S, Sieghart W. Comparative modeling of $GABA_A$ receptors: limits, insights, future developments. Neuroscience 2003;119:933–43.
[28] Berezhnoy D, Nyfeler Y, Gonthier A, Schwob. Goeldner M, Sigel E. On the benzodiazepine binding pocket in $GABA_A$ receptors. J Biol Chem 2004;279:3160–8.
[29] Kash TL, Jenkins A, Kelley JC, Trudell JR, Harrison NL. Coupling of agonist binding to channel gating in the $GABA_A$ receptor. Nature 2003;421:272–5.
[30] Teissere JA, Czajkowski C. A β-strand forms part of the benzodiazepine binding site of the GABA–A receptor: structural rearrangements detected during channel activations. J Neurosci 2001;21: 4977–86.
[31] Yamakura T, Bertaccini E, Trudell JR, Harris RA. Anesthetics and ion channels: molecular models and sites of actions. Ann Rev Pharmacol Toxicol 2001;41:23–51.
[32] Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, et al. Sites of alcohol and volatile anesthetic action on $GABA_A$ and glycine receptors. Nature 1997;389:385–9.
[33] Scheller M, Forman SA. The y subunit determines whether anesthetic-induced leftward shift is altered by a mutation at α1S270 in α1β2γ2L $GABA_A$ receptors. Anesthesiology 2001;95:123–31.
[34] Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, et al. Evidence for a common binding cavity for three general anesthetics within the $GABA_A$ receptor. J Neurosci 2001;21:RC136.
[35] Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, et al. Mutations of GABA and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? Proc Natl Acad Sci USA 1998;95:6504–9.
[36] Mascia MP, Trudell JR, Harris RA. Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. Proc Natl Acad Sci USA 2000;97:9305–10.
[37] Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, Harrison NL. Propofol and other intravenous anesthetics have sites of action on the $GABA_A$ receptor distinct from isoflurane. Mol Pharmacol 1998;53:530–8.
[38] Rick CE, Ye Q, Finn SE, Harrison NL. Neurosteroids act on the $GABA_A$ receptor at sites on the N-terminal side of the middle of TM2. NeuroReport 1998;9:379–83.
[39] Amin J. A single hydrophobic residue confers barbiturate sensitivity to $GABA_C$ receptor. Mol Pharmacol 1999;55:411–23.
[40] Serafini R, Bracamontes J, Steinbach JH. Structural domains of the human $GABA_A$ receptor β3 subunit involved in the actions of pentobarbital. J Physiol 2000;524(3):649–76.
[41] Cestari IN, Min KT, Kulli JC, Yang J. Identification of an amino acid defining the distinct properties of murine β1 and β3 subunit-containing $GABA_A$ receptors. J Neurochem 2000;74:827–38.
[42] Belelli D, Lambert JJ, Peters JA, Wafford K, Whiting PJ. The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. Proc Natl Acad Sci USA 1997;94:11031–6.
[43] Moody EJ, Knauer C, Granja R, Strakhova M, Skolnick P. Distinct loci mediate the direct and indirect actions of the anesthetic etomidate at $GABA_A$ receptors. J Neurochem 1997;69:1310–3.
[44] Quinlan JJ, Homanics GE, Firestone LL. Anesthesia sensitivity in mice that lack the β3 subunit of the $GABA_A$ receptor. Anesthesiology 1998;88:775–80.
[45] Carlson BX, Engblom C, Kristiansen U, Schousboe A, Olsen RW. A single glycine residue at the entrance to the first membrane-spanning domain of the $GABA_A$ receptor β2 subunit affects allosteric sensitivity to GABA and anesthetics. Mol Pharmacol 2000;57:474–84.
[46] Engblom AC, Carlson BX, Olsen RW, Schousboe A, Christiansen U. Point mutation in the first trans-membrane region of the β2 $GABA_A$ subunit alters desensitization kinetics of GABA- and anesthetic-induced channel gating. J Biol Chem 2002;277:17438–47.
[47] Chang CS, Olcese R, Olsen RW. A single M1 residue in the beta2 subunit alters channel gating of $GABA_A$ receptor in anesthetic modulation and direct activation. J Biol Chem 2003;278:42821–8.
[48] Wallner M, Hanchar HJ, Olsen RW. Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci USA 2003;100:15218–23.
[49] Hanchar HJ, Wallner M, Olsen RW. Revealing the molecular determinants responsible for differences in ethanol sensitivity of β1, β2, and β3 containing α4/6βδ $GABA_A$ receptors. Alcohol Clin Exp Res 2004;28(Suppl):91A [RSA Abstract 508].
[50] Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. $GABA_A$receptors: immunocytochemical distribution of 13 subunits in the adult brain. Neuroscience 2000;101:815–50.

[51] Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, et al. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. Proc Natl Acad Sci USA 2004;101:3662–7.

[52] Stell BM, Brickley SG, Tang CY, Farrant M, Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci USA 2003;100:14439–44.

[53] Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA. Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. Br J Pharmacol 2002;136:965–72.

[54] Wohlfarth KM, Bianchi MT, Macdonald RL. Enhanced neurosteroid potentiation of ternary $GABA_A$ receptors containing the δ subunit. J Neurosci 2002;22:1541–8.

[55] Wallner M, Hanchar HJ, Olsen RW. Extrasynaptic GABA(A) receptors as mediators of low concentration ethanol effects. Alcohol Clin Exp Res 2004;28(Suppl):92A [RSA Abstract 515].

[56] Bianchi MT, Macdonald RL. Neurosteroids shift partial agonist activation of GABA(A) receptor channels from low- to high-efficacy gating patterns. J Neurosci 2003;23:10934–43.

[57] Korpi ER, Kleingoor C, Kettenmann H, Seeburg PH. Benzodiazepine-induced motor impairment linked to point mutation in cerebellar $GABA_A$ receptor. Nature 1993;361:356–69.

[58] Wieland H, Luddens H, Seeburg PH. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 1992;257:1426–9.

[59] Hanchar HJ, Wallner M, Olsen RW. The ANT mutation links alcohol hypersensitive rats and alcohol supersensitive $GABA_A$ receptors. Abstr Soc Neurosci 2004.

[60] Xi J, Liu R, Asbury GR, Eckenhoff MF, Eckenhoff RG. Inhalational anesthetic-binding proteins in rat neuronal membranes. J Biol Chem 2004;279:19628–33.

[61] Chiara DC, Dangott LJ, Eckenhoff RG, Cohen JB. A binding site for the volatile anesthetic halothane within the nicotinic acetylcholine receptor identified by photoaffinity labeling. Biochemistry 2003;42:13457–67.

[62] Smith SS, editor. Neurosteroids and the $GABA_A$ receptor. Boca Raton, FL: CRC Press; 2003.

[63] Darbandi-Tonkabon R, Hastings WR, Zeng CM, Akk G, Manion BD, Bracamontes JR, et al. Photoaffinity labeling with a neuroactive steroid analogue. 6-Azi-pregnanolone labels voltage-dependent anion-channel-1 in rat brain. J Biol Chem 2003;278:13196–206.

[64] Darbandi-Tonkabon R, Manion BD, Hastings WR, Craigen WJ, Akk G, Bracamontes JR, et al. Neuroactive steroid interactions with voltage-dependent anion channels: lack of relationship to $GABA_A$ receptor modulation and anesthesia. J Pharmacol Exp Ther 2004;308:502–11.

[65] Husain S, Forman SA, Kloczeweiak MA, Addona GH, Olsen RW, Pratt MB, et al. Synthesis and properties of 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine, a photoactivatable general anesthetic. J Med Chem 1999;42:3300–7.

[66] Pratt MB, Husain SS, Miller KW, Cohen JB. Identification of sites of incorporation in the nicotinic acetylcholine receptor of a photoactivatible general anesthetic. J Biol Chem 2000;275:29441–51.

[67] Ruesch D, Zhong H, Forman SA. Gating allosterism at a single class of etomidate sites on $\alpha1\beta2\gamma2L$ $GABA_A$ receptors accounts for both direct activation and agonist modulation. J Biol Chem 2004; 279:20982–92.

[68] Husain SS, Ziebell MR, Ruesch D, Hong F, Arevalo E, Kosterlitz J, et al. 2-(3-Methyl-3*H*-diaziren-3-yl)ethyl 1-(1-phenethyl)-1*H*-imidazole-5-carboxylate: a photoactivatable stereoselective intravenous general anesthetic probe for ligand-gated ion channels. J Med Chem 2003;46:1257–65.

[69] Ziebell MR, Nirthanan S, Husain SS, Miller KW, Cohen JB. Identification of binding sites in the nicotinic acetylcholine receptor for [^{3}H]azietomidate, a photo-activatable general anesthetic. J Biol Chem 2004;279:17640–9.

[70] Sawyer GW, Miller KW, Husain SS, Olsen RW. Photolabeling of $GABA_A$ receptor with a radioactive anesthetic azi-octanol. Abstr Soc Neurosci 2000;26:1335. #500.3.

[71] Li G, Sawyer GW, Husain SS, Olsen RW. $GABA_A$ receptor subunits were photoaffinity labeled with [^{3}H] azietomidate. Abstr Soc Neurosci 2004.

[72] Trudell JR. Unique assignment of inter-subunit association in $GABA_A$ alphal beta3 gamma2 receptors determined by molecular modeling. Biochim Biophys Acta 2002;1565:91–6.