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$GABA_A$ Receptor: Positive and Negative Allosteric Modulators

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Abstract

gamma-Aminobutyric acid (GABA)-mediated inhibitory neurotransmission and the gene products involved were discovered during the mid-twentieth century. Historically, myriad existing nervous system drugs act as positive and negative allosteric modulators of these proteins, making GABA a major component of modern neuropharmacology, and suggesting that many potential drugs will be found that share these targets. Although some of these drugs act on proteins involved in synthesis, degradation, and membrane trasnsport of GABA, the GABA receptors Type A (GABAAR) and Type B (GABABR) are the targets of the great majority of GABAergic drugs. This discovery is due in no small part to Professor Norman Bowery. Whereas the topic of GABABR is appropriately emphasized in this special issue, Norman Bowery also made many insights into GABAAR pharmacology, the topic of this article. GABAAR are members of the ligand-gated ion channel receptor superfamily, a chloride channel family of a dozen or more heteropentameric subtypes containing 19 possible different subunits. These subtypes show different brain regional and

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Chemical compounds, using PubChem CID:#.

GABA;	119
muscimol,	4266
bicuculline,	10237
diazepam,	3016
Ro15-4513,	5081
picrotoxinin,	442292
TBPS,	104781
pentobarbital,	4737
alphaxalone,	104845
etomidate,	667484

Competing interests
None

Conflicts of interest
None

Human and animal rights
Not applicable

subcellular localization, age-dependent expression, and potential for plastic changes with experience including drug exposure. Not only are GABAAR the targets of agonist depressants and antagonist convulsants, but most GABAAR drugs act at other (allosteric) binding sites on the GABAAR proteins. Some anxiolytic and sedative drugs, like benzodiazepine and related drugs, act on GABAAR subtype-dependent extracellular domain sites. General anesthetics including alcohols and neurosteroids act at GABAAR subunit-interface trans-membrane sites. Ethanol at high anesthetic doses acts on GABAAR subtype-dependent trans-membrane domain sites. Ethanol at low intoxicating doses acts at GABAAR subtype-dependent extracellular domain sites. Thus GABAAR subtypes possess pharmacologically specific receptor binding sites for a large group of different chemical classes of clinically important neuropharmacological agents.

Keywords

γ-Aminobutyric acid type A receptors; inhibitory neurotransmission; CNS depressant drug target; anxiolytics/sedatives/general anesthetics; ethanol

1. INTRODUCTION

The concept of receptors as biochemical mediators of cell signaling including the actions of neurotransmitters has existed for over 100 years, coming to an era of respect for receptors' existence with the advent of radioligand binding in the 1960's and '70's, and a major sub-discipline of pharmacology in the birthplace and world center of pharmacology, the United Kingdom (Rang, 1973; Cuatrecasas, 1974). Proteins were established as neurotransmitter receptors, notably for established neurotransmitters like acetylcholine (Changeux, 1981) and catecholamines (Pepeu et al., 1980) and drugs like opiates that turned out to be ligands for receptors of endogenous neurotransmitters (Snyder, 2017). The recognition of amino acids (Curtis and Johnston, 1974) including glutamate (Watkins and Evans, 1981) and γ–aminobutyric acid (GABA)* as major neurotransmitters proceeded during the '60's and 70's (Eccles, 1969), culminating in a meeting organized by Eugene Roberts in 1975 at the Kroc Foundation's Golden Arches Ranch outside Santa Barbara, California, with classic presentations and reviews published by world leaders (Roberts et al., 1976). But the real beginning of the GABA era occurred at a world-wide meeting of virtually all interested parties organized by Frode Fonnum in Spatind, Norway in 1977 (Fonnum, 1978; Dray & Bowery, 1978; Olsen et al., 1978). This was where most of the players who developed the GABA field in subsequent years first gathered and met, including Norman Bowery.

Just beginning work on GABA, I found Norman a pioneer, publishing in the late 70's on $GABA_A$ receptor ($GABA_AR$) positive and negative allosteric modulators (PAMs and NAMs) that will be the emphasis of this chapter. Yes, Norman made many contributions to the GABA field including GABA transporters and $GABA_ARs$ besides $GABA_B$ receptor work! I visited him in London in early 1978 at the School of Pharmacy. Not only did he keep me

*Abbreviations: GABA: γ-aminobutyric acid: GABAAR: GABAA receptors; LGIC: ligand-gated ion channels; PLGIC: pentameric LGIC; CNS: central nervous system; ECD: extracellular domain; TMD: trans-membrane domain; GlyR' glycine receptors; TM1: trans-membrane helix 1;TM2: trans-membrane helix 2; TM3: trans-membrane helix 3; SCAMP: Substituted cysteine modification protection

excited about GABA for an hour, but his whole department of 10 also talked to me only about GABA. I felt it was like heaven, and, considering the outstanding pharmacology community in England, including GABA workers, I hastily arranged a sabbatical leave in London for the following year at the National Institute for Medical Research, Mill Hill, with numerous jovial, valuable visits with Norman.

The excitement of these times (late '70's to early '90's) in neuropharmacology was due to the new clues to understanding the molecular mechanisms of action of signaling systems like neurotransmission, but also neuropharmacological agents important in humans, notably the anxiolytic, anticonvulsant benzodiazepines, picrotoxin-like convulsants, general anesthetics including both volatile agents (like isoflurane), and intravenous agents: barbiturates, etomidate, propofol, as well as long-chain alcohols, including low potency actions of EtOH, plus neuroactive steroids. All are GABAAR PAMs and NAMs. Furthermore, at another site on $GABA_A$Rs, high potency (low mM) EtOH actions are also shown to be due to action as a $GABA_A$R PAM. Norman Bowery was one of the leaders in recognizing that the field possessed valuable tools in the libraries of chemical compounds that exhibit pharmacological efficacy: these are ligands, not only for the isosteric GABA binding sites on the critical macromolecules (transporters and receptors) (Bradley and Dray, 1973; Bowery, 1984; Tanaka and Bowery, 1996; Ejeberg et al., 2002; Johnston, 2000; Martin and Olsen, 2000; Olsen, 2014), but also ligands for allosteric sites on those same neurotransmission proteins (Haefely et al., 1975; Ticku and Olsen, 1980; Supavilai et al., 1982b; Johnston, 1984; Harrison and Simmonds, 1984).

2. GABA and $GABA_A$R

In this special issue for Norman Bowery with emphasis on $GABA_B$ receptors, the importance of GABA in brain function, and the pharmacology, structure and function of $GABA_B$ receptors (Bowery et al., 2002; Pin and Bettler, 2016) will be sufficiently covered. Unlike the $GABA_B$ receptors, which are members of the relatively slow, G-protein-coupled receptors (GPCR) category, GABAARs are part of the rapid-acting, ligand-gated ion channel (LGIC) receptor category. They are members of the pentameric (PLGIC) superfamily, which includes nicotinic acetylcholine, 5-HT3, and inhibitory glycine receptors, which differ from the tetrameric ionotropic glutamate receptors and trimeric purinergic subclass of LGIC (Collingridge et al, 2009). The PLGIC superfamily has many structural features in common, including homologous subunit sequences and functional domains within the heteropentameric proteins (Galzi and Changeux; 1994; Harvey and Betz, 2000; Olsen and Sieghart, 2008). Many are understood at the high (atomic) resolution structural level, based on both X-ray crystal structures of model homopentameric proteins (see below), as well as computer-enhanced images generated from electron microscopy (Unwin, 2005) and cryo-electron microscopy, e.g., TRK (Julius, 2013), NMDA (Lu et al., 2017), and the closely related PLGIC glycine receptor (Du et al., 2015). The latter technology could be applied to both the structure of the GABAAR channel, and location of the functional ligand-binding sites, and the allosteric modulatory sites on native heteromeric membrane proteins, including subtypes, as well as plastic changes occurring in vivo, as in learning and memory, and chronic drug treatment. Other developments are erupting from the use of high resolution

proteomics to identify partners for protein–protein interactions, e.g., for GABABRs (Schwenk et al, 2016) and GABAARs (Yamasaki et al., 2017).

The $GABA_A$Rs are constituted from a family of 19 homologous genes categorized by degree of sequence identity as different subunit families (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3). They are usually constructed with 2 copies of an α subunit, 2 copies of a β subunit, and one copy of either a γ subunit, or another such as δ, to form a family of perhaps 2 dozen heteropentameric subtypes, about one dozen of which are sufficiently abundant to have physiological relevance (Barnard et al., 1988; Olsen and Sieghart, 2008). These vary in age and brain regional as well as subcellular distribution, and thus in functional, circuitry involvement, regulatory aspects, and pharmacology, depending on the subunit composition, and involving minor structural heterogeneity within a common basic structure and function. The heterogeneity provide the opportunity for possible subtype-selective pharmacological ligand specificity and clinical therapeutics with minimal side effects (Whiting, 1993; Hevers and Lüddens, 1998; Möhler et al., 2002).

$GABA_A$Rs and $GABA_B$ receptor proteins differ greatly from each other, including the GABA binding site domains, so that the pharmacology of isosteric analogues of GABA are different for the two: muscimol (agonist) and bicuculline (antagonist) are specific $GABA_A$R ligands, while baclofen is a specific $GABA_B$ receptor agonist (Bowery, 1984; Bowery et al., 2002). Despite earlier suggestions to the contrary, both types of GABA receptor participate in both postsynaptic inhibition and presynaptic inhibition. There is some evidence that presynaptic and postsynaptic inhibition by GABAB receptors involves different subtypes (Bowery et al., 1980). Similarly, it is likely that presynaptic and postsynaptic inhibition by $GABA_A$Rs will be demonstrated to be a function of different subtypes. Both GABAA and GABAB receptors also exist at both synaptic and extrasynaptic locations.

$GABA_A$Rs demonstrate considerable extrasynaptic localization where they function to mediate ‘tonic’ inhibitory currents, distinguishable from ‘phasic’ synaptic transmission (Bai et al., 2001; Mody and Pearce, 2004; Farrant and Nusser, 2005). The extrasynaptic $GABA_A$Rs are primarily due to the α4, α5, and α6 subunit-containing subtypes, while the α1, α2, and α3 subtypes are sometimes extrasynaptic but constitute the postsynaptic GABAARs (Fritschy et al., 1992; Hevers and Lüddens, 1998; Olsen and Sieghart, 2008). GABA signaling has been widely implicated in mechanisms of drug addiction (e.g., Olsen and Liang, 2017). Currently, in addition to CNS functions, the GABA described in many peripheral tissues for over 50 years has been implicated increasingly in physiological roles and disorders, such as diabetes (Tian et al., 2017; Li et al., 2017), inflammatory disease including regulation of immune cell proliferation (Tian et al., 2004), stem cell proliferation (Urrutia et al., 2016), and stimulation of cell energy metabolism in the cardiovascular system, including cardiac myocytes (Lorente et al., 2000; Zhang et al., 2002) and endothelial cells (Sen et al., 2016), where the authors provide evidence that the GABA made and released by these cells may originate from putrescine rather than glutamate and the usual neuronal mechanism involving L-glutamate decarboxylase.

2.1. $GABA_AR$ Ligand Sites

$GABA_ARs$ were characterized initially for three major drug sites: the GABA sites (agonist/antagonist) in the extracellular domain (ECD); the picrotoxin sites (channel blocker; here defined also as a negative allosteric modulator, NAM), in the transmembrane domain (TMD); and the benzodiazepine (BZ) sites (positive allosteric modulator, PAM) in the ECD (Usdin et al., 1982). Radioligand binding was utilized to identify and characterize the ligand binding sites. I will summarize the GABA and BZ and picrotoxin sites briefly, and pay more attention to two other classes of sites, the anesthetic sites discovered biochemically by allosteric modulation of the three radioligand binding sites, especially the picrotoxin site, and identified in the TMD by affinity labeling, and the 'high affinity' ethanol site on extrasynaptic δ subunit-containing $GABA_ARs$, which is a modified BZ binding site, which in turn is a modified GABA binding site in the ECD; these latter two sites for allosteric modulatory ligands with low affinity and specificity were technically more difficult to study biochemically.

Fig. 1 (Olsen, 2015; Wallner et al., 2017) shows a schematic view of the heteropentameric $GABA_AR$ protein(s) with ligand binding sites positioned throughout and described over the course of this article. It is based on X-ray crystal structure and electron microscope images of PLGIC proteins including some partial $GABA_AR$ proteins. The positions suggested for the GABA and BZ binding sites by mutagenesis and affinity labeling have been confirmed using homology models of the ECD of the Acetylcholine Binding Protein, AChBP (Brejc et al., 2001; Cromer, et al., 2002; Ernst et al., 2005). It had many similarities to the computer-enhanced electron microscope image of *Torpedo marmorata* nicotinic acetycholine receptor, include membrane-spanning domains (TMD) (Unwin, 2005). Several pentameric ligand-gated ion channels (PLGICs) have been solved at high resolution: the prokaryotic *Erwinia chrysanthemi* (ELIC) (Hilf and Dutzler, 2008), the *Gloeobacter violaceus* homologue (GLIC) (Bocquet et al., 2009; Corringer et al., 2010), the glutamate-gated anion-selective Cys-loop receptor GluCl found in nematode *C. elegans* (Hibbs and Gouaux, 2011), and a homopentameric human β3-$GABA_AR$ with several flexible domains deleted by genetic engineering (Miller and Aricescu, 2014). Despite great progress, we are still lacking structural information of heteropentameric $GABA_ARs$ with ligands (BZ, anesthetics) docked into binding sites at extracellular and transmembrane subunit interfaces (see below). Homology models using multi-template composites identified further putative binding sites for $GABA_AR$ allosteric ligands (Puthenkalam et al., 2016).

2.1.1. The GABA Sites—$GABA_AR$-specific radioligand binding to detect and characterize receptors was achieved by Sol Snyder's lab, with labeled GABA itself, using Na^+-independent buffer for assay to prevent GABA binding to the uptake sites on cell membranes in brain (Zukin et al., 1974), and later the specific ligand muscimol was used by two former Snyder postdocs, Sam Enna and Hank Yamamura (Beaumont et al., 1980). The drug muscimol, isolated from mushrooms *Amanita muscaria*, and analogues were characterized by Krogsgaard-Larsen et al., 1975), including THIP (gaboxadol: Krogsgaard-Larsen et al., 1977) as $GABA_AR$ agonists, supplemented with antagonists bicuculline (natural product: Johnston, et al., 1972) and GABAzine (synthetic [SR95531, gabazine]: Wermuth and Biziere, 2002) as antagonists. Identification of *$GABA_AR$-specific* binding was

made possible by comparison of the in vitro and in vivo potencies of a series of analogues of GABA, among them compounds of restricted conformational flexibility/increased rigidity, both naturally occurring and specifically synthesized as tools (Johnston et al., 1975; Olsen et al., 1978; Enna and Snyder, 1975).

The GABA binding sites were located in the ECD of the receptor protein by affinity labeling and site-directed mutagenesis (Amin and Weiss, 1993; Smith and Olsen, 1994) to the interface between α and β subunits (two per pentamer); the multiple small 'loops' on each of the interface subunits were compatible with, and then shown to involve, small stretches of β-sheets (Boileau et al., 1999). That portion of the site containing the defined loops 'A, B, and C' were on the β subunit, designated the '+' subunit, while the loops 'D, E, and F' were on the α subunit, designated '−', defining the GABA site at the 'β+/α−' interface (Fig. 1, Olsen, 2014; Olsen and Sieghart, 2008). The two GABA sites were not identical in 3-dimensional structure, and exhibited cooperativity in binding kinetics, but virtually identical chemical specificity for ligands (Changeux and Edelstein 2011). Small but significant differences in agonist and antagonist sensitivity were demonstrated for subtypes differing in subunit composition, interestingly, the presence of a γ or δ subunit altered these ligand selectivities compared to no γ or δ, and from each other (Barnard et al., 1998; Hevers and Lüddens, 1998). In particular the δ subunit imparted higher affinity for many GABA agonists, including GABA, muscimol, and gaboxadol, while the γ subunit imparted lower affinity (Meera et al., 2011).

2.1.2. The Benzodiazepine (BZ) Sites—BZs were some of the most famous and successful of all drugs, ever. They have been widely used for anxiety, panic attacks, some types of epilepsy, muscle relaxation, pre-anesthesia, and sleep (Haefely,1989). They continue to be used to some extent, but suffer from numerous problems: tolerance for the most desired effects, multiple side-effects (as PAMs for $GABA_A$Rs, they act on all brain circuits and functions, normal and abnormal), and they produce physical and psychological dependence, with severe withdrawal syndromes (Trimble, 1983).This dependence aspect of chronic use and over-use continues to affect many patients today (Lader, 2014).

Identification of the 'BZ receptors' binding sites was accomplished by Braestrup and Squires (1977) and Möhler and Okada (1977) with [^{3}H]diazepam binding. BZ drugs were CNS depressants and shown to act as PAMs on $GABA_A$Rs (Costa and Guidotti, 1979; Haefely, 1982). BZ receptor binding was shown to be enhanced by GABA in the test tube (Karobath et al., 1979; Tallman et al., 1980), because the BZ sites are situated on the $GABA_A$R proteins, and allosterically coupled to the GABA sites (Stephenson and Olsen, 1982). This was verified biochemically by solubilizing and purifying one protein containing both GABA, BZ, and modulatory sites (Gavish and Snyder, 1980; Sigel and Barnard, 1984; Supavilai and Karobath, 1984; King et al., 1987), as well as cloning of the BZ site-containing $GABA_A$R protein's genes (Schofield et al., 1987).

Heterogeneity of BZ sites in different brain regions was evident from the beginning, with some chemical classes of ligands varying in potency (Lo et al., 1983; Olsen et al., 1991). Ligands for BZ and picrotoxin sites also allosterically modulate each other (Olsen, 1982; Supavilai et al., 1982), consistent with multiple allosterically coupled sites on a single

protein complex, the $GABA_AR$-chloride channel. This helped establish that these drugs acted on the brain via PAM action on $GABA_ARs$. But the degree of modulation also showed regional heterogeneity. This suggested presumably circuitry and behavioral distinctions consistent with pharmacological subtypes, and the potential development of new drugs with subtype selectivity and hopefully improved clinical profiles (Barnard et al., 1998; Lüddens et al., 1995; Olsen and Sieghart, 2008; Rudolph and Möhler, 2004; Whiting et al., 1995). Despite heroic achievements on understanding the actions of these drugs, the normal functions affected and diseases treated, with brilliant advances in analyzing $GABA_AR$ subtype functions and their roles in disorders and therapeutics, using modern techniques including genetic engineering (Rudolph et al., 1999; McKernan et al., 2000; Low et al., 2000), development of suitable disease-specific BZ-site-directed clinically used drugs has been limited, but hope springs eternal (Möhler et al., 2002).

Interestingly, the BZ binding sites in the ECD of the $GABA_AR$ protein, similarly identified by site-directed mutagenesis (Amin et al., 1997; Teissere and Czjakowski, 2001; Sigel, 2002) and affinity labeling (Möhler et al., 1980; Sieghart and Karobath, 1980; Benke et al., 1996; Duncalfe et al., 1996, Smith and Olsen, 2000) turned out to be in sequence loops that were, like the GABA sites, based on β-sheet structures, and located at the α+/γ− interface (γ subunits are required for BZ binding and sensitivity, Pritchett et al., 1989). Moreover, the sequence loops in the α subunit BZ binding domain resemble in amino acid sequence those in the β subunit GABA binding domain (Galzi and Changeux, 1994; Smith and Olsen, 1995; Sigel and Buhr, 1997). In other words, not only is the potent exogenous modulator of the brain acting on an allosteric modulatory site of $GABA_ARs$ but this site is a modified binding site for the neurotransmitter ligand GABA (located on α+/γ− rather than β+/α− interface in the same protein). In the vernacular of this field, BZs are actually co-agonists of GABA (not actually mimicking the action at the same identical site as GABA, but at an homologous site, with remarkable potency (Forman and Miller, 2011). Further, one can have synergistic activation of $GABA_ARs$; note that BZs can act as agonists in the absence of GABA (Akk et al., 2017; Campo-Soria, Chang, and Weiss, 2006; Rüsch and Forman, 2005), but only at higher concentrations and will low efficacy, so not important to human pharmacology. Conventional wisdom categorizes BZs as PAMs (Haefely, 1989; Barnard et al., 1998). BZ ligands also exhibit variable efficacy, the GABAAR enhancement considered agonist efficacy, while partial agonists (Braestrup et al., 1979) and inverse agonists (blockers of GABAAR: Braestrup et al., 1982) also occur, as well as antagonists (block agonists and inverse agonists without intrinsic efficacy on GABAAR function: (Hunkeler et al., 2012)).

The BZ pharmacology at $GABA_ARs$ are of major interest in GABA neuropharmacology, but for this story, suffice it to say here, that the γ2 subunit is most abundant in all $GABA_AR$ subtypes, so imparts BZ sensitivity to the majority of $GABA_AR$ subtypes, as well as the opportunity for these subtypes to be synaptically localized (Lüddens et al., 1995). The δ-containing subtypes are relatively low abundance, other γ subunits are sensitive to most BZ, but quite rare in abundance, and the subunits replacing γ and δ, such as ε, even more rare (Olsen and Sieghart, 2008). Thus there is major heterogeneity in BZ sensitivity of $GABA_ARs$: the α4 and α6 subunits, coupled with γ2 subunits, potently bind many antagonist and inverse agonist BZ ligands, but not the traditional BZ agonists like diazepam, thus producing diazepam-insensitive (DZ-IS) binding sites (Hevers and Lüddens, 1998).

These α4 and α6 $GABA_AR$ subtypes, besides the γ2-containing versions, also partner with δ subunits, producing extrasynaptic $GABA_ARs$ that mediate tonic inhibitory currents in major cell populations including cerebellar and hippocampal granule cells (Farrant and Nusser, 2005; Olsen and Sieghart, 2008). They were thought not to bind any BZ ligands, lacking the high affinity α+/γ− ("site 1"), but then were found to bind some BZ ligands with lower affinity at distinct other sites on the $GABA_AR$ proteins. Here we define BZ "site 2" and "site 3". These 'non-traditional' sites may mediate pharmacological effects of BZ ligands on GABAAR subtypes that are insensitive to traditional BZ agonists, and thus circuits and functions not traditionally considered BZ targets. For the most part, these discoveries are limited to use of novel ligands as research tools, although hopefully some clincially relevant compounds emerge.

While Site 1 uses specific protein loop sequences in α subunits (Buhr et al., 1997) that are adjacent to γ2 subunits, Site 2 uses the same protein loops in the other α subunit, at an homologous interface with a β subunit, to form a second type of BZ site, similar but different from site 1 (Baur et al., 2008; Maldifassi et al., 2016).This site 2 can be present in $GABA_AR$ subtypes with γ2 or those lacking γ2 subunits, either with no replacement or with δ (Ramerstorfer et al., 2011; Wallner et al., 2014). The pharmacology seems significantly different from site 1. Pyrazoloquinolinones are BZ site-active PAMs in γ–containing subtypes, and show distinct variability in efficacy, as well as α and β subunit selectivity, in other words, BZ-site ligands that have more or less efficacy than traditional BZ agonists on the traditional BZ-sensitive subtypes, and unexpected efficacy on the diazepam-insensitive subtypes like α4- or α6-containing, or αβ without γ subtypes (Varagic et al., 2013; Simeone et al., 2017). In the case of diazepam-insensitive δ-containing subtypes, my lab found greater sensitivity (low mM) to EtOH for PAM efficacy, involving site 2 binding of selected BZ ligands including EtOH antagonists like Ro15-4513 (Hanchar et al., 2006; Wallner et al., 2014), described below.

Site 3 for BZ binding (or perhaps not binding but allosteric coupling to modulatory efficacy) was suggested based on low potency BZ pharmacological effects in vivo and demonstrated in vitro (Walters et al., 2000; Middentorp et al., 2015). These appear to be located in the TMD at sites also implicated for various PAMs like general anesthetics (Belelli et al., 1997; Rudolph and Antkowiak, 2004), as proven by mutagenesis and in vivo analysis of genetically engineered mice (Jurd et al., 2003; Reynolds et al., 2003; Antkowiak and Rudolph, 2016). This site 3 (or sites) at five TMD interfaces is also discussed in more detail below. Note that some ligands clearly overlap on more than one type of site on these remarkably promiscuous, while still quite specific, drug receptors.

Sieghart et al. (2012) and Sieghart (2015) present evidence that the GABA-preferring β+/α− sites also exhibit measureable low affinity for some BZ ligands like flurazepam and this could be accompanied by weak pharmacological efficacy (site 4?). Finally, we have shown that the BZs have some low affinity for the picrotoxin site described below (Site 5?), probably explaining the convulsant efficacy of the flurazepam analogue Ro5-3663 (Leeb-Lundberg et al., 1981b; Olsen, 1986).

2.1.3. The Picrotoxin Site—Picrotoxin, a natural plant product, with universal efficacy as blocker of $GABA_AR$ chloride channels, is a molecular pair of two isomers: the active picrotoxinin and the much less active picrotin; the $GABA_AR$ channel blocking activity is shared by some close structural analogues found in related plants, such as tutin in New Zealand 'loco-weed' (makes cows sick with bizarre behaviors) (Jarboe et al., 1968; Ticku and Olsen, 1980). Not a chemical analogue of GABA, it does not bind to the GABA recognition site in the ECD, but rather to residues in the TMD channel pore (Olsen, 1982). Speculated to physically obstruct the channel (Takeuchi and Takeuchi, 1969), picrotoxin needed agonist binding and opening of the channel to enable channel block (Macdonald and Olsen, 1994; Sieghart, 2015).

For the purposes of this review, we note that by definition, picrotoxin is a NAM. Clearly it blocks GABA-activated chloride flux at another (allosteric) site than the GABA binding site. But one could say the functional part of the GABAAR molecule is the (picrotoxin) site-ion channel, and the GABA site is the regulatory site, allosteric to the channel. If picrotoxin acts as a channel blocker, then we have defined how picrotoxin acts as a NAM to GABA and we don't' need to call it a NAM, but rather, a channel blocker. The word allosteric has come to be used mainly for conformational change effects for ligands binding elsewhere on the protein than the active site rather than a physical obstruction (or enhancement) of function, but this depends on your definitions. GABAARs are complicated, and developing structural information helps to clarify the terminology (Christopoulos et al., 2014).

We synthesized a radiolabeled analogue [^{3}H]dihydropicrotoxinin (DHP) and demonstrated specific receptor binding sites in membrane homogenates of crayfish muscle and mammalian brain (Olsen et al., 1978; Ticku et al., 1978; Leeb-Lundberg et al., 1980). This provided another tool for characterizing $GABA_ARs$, with the very important bonus of finding pharmacologically specific inhibition of binding by some convulsants with unknown mechanisms of action, and allosteric modulation, positive or negative, by numerous neuroactive drugs suspected of GABAergic mechanisms, such as general anesthetics (Olsen et al., 1979). [^{3}H]DHP binding allowed discovery of a wealth of ligands which competitively or allosterically modulated picrotoxin binding when their pharmacological effect on organisms, cells, and channels involved $GABA_ARs$. Clearly the agents binding to the picrotoxin site on $GABA_AR$ had activity as PAMs or NAMs on $GABA_AR$ function and pharmacology in vivo.

The binding was inhibited by several "cage convulsants" (Ticku and Olsen, 1979), previously demonstrated to be GABAAR noncompetitive inhibitors (NAMs by our definition, but probably picrotoxin site ligands, which may be channel blockers) (Bowery et al., 1976). These cage convulsants (trioxabicyclo-octanes) were synthesized as potential pesticides acting on the nervous system (Casida, 1993), including some highly toxic to vertebrates, such as *t*-butyl bicyclophosphorothionate (TBPS). This ligand [^{35}S]TBPS was a superior high affinity ligand tool for the picrotoxin site; the binding was found to be allosterically inhibited by GABA agonists, and crude homogenates of tissues/cells containing GABAARs had to be washed free of endogenous GABA before binding could be detected (Squires et al., 1983). Binding to these sites was inhibited by thujone, a possible stimulant identified in the classical *fin de siecle/Gay 90's* beverage absinthe (Hold et al.,

2000), regarded by many as a fount of creativity (Olsen, 2000). Further, many insecticides, e.g., lindane, dieldrin, and fipronil (Hosie et al., 1995; Ikeda et al., 2001; Chen et al., 2006; Olsen, 2006), were found to inhibit the picrotoxin/TBPS site in GABAARs and mimic their pharmacology. Other convulsants considered to act on these sites included pitrazepin and tetramethylenedisulfotetramine (TETS) (Bowery et al., 1975; Zhao et al., 2014), including agents used as human poisons (Whitlow et al., 2005).

Studies combining the pharmacology of these insecticides dieldrin and lindane with the molecular characterization of insect $GABA_AR$ chloride channels helped establish the channel site of action for picrotoxin and the insecticides. The isolation of strains of insects resistant to these insecticides and demonstration that a single gene responsible for this resistance, called *rdl* for 'resistance to dieldrin', had a single amino acid mutation relative to the wild type sequence; this gene product was the insect equivalent of $GABA_ARs$, and the mutation was in the TM2 region corresponding to the chloride channel in the TMD (ffrench-Constant et al., 1993). The binding site was shown earlier to be located physically on the mammalian GABAAR protein (Sigel and Barnard, 1984; Supavilai and Karobath, 1984; King et al., 1987).

Additionally, mutagenesis of the amino acid implicated in dieldrin resistance in the *rdl* mutant (GABAAR β subunit) to a cysteine allowed an insecticide analogue modified with a sulfhydryl reagent moiety to bind covalently (affinity label) the channel at this residue, so-called proximity-accelerated covalent coupling (PACC: Perret et al., 1999); the attachment of the insecticide affinity label produced an irreversibly inhibited channel, and block of the picrotoxin/TBPS binding sites. Homology structural modeling and molecular dynamics simulations showed that the different toxin ligands bind at overlapping but non-identical sites in the protein channel that indeed vary with subunit subtypes (Zhao et al., 2014). This work demonstrated conclusively that the picrotoxin site was on the GABAAR protein, located apparently in the channel, at a site distinct from the GABA site.

Several members of the PLGIC family actually have some affinity for picrotoxin and related ligands, including primarily some subtypes of inhibitory chloride channel glycine receptors (Pribilla et al., 2010) and the nematode GluCl PLGIC protein. The X-ray crystal structure of GluCl protein with picrotoxinin showed the ligand bound within the channel (Hibbs and Gouaux, 2011), consistent with models of the GABAAR (Olsen et al.; 2014; Zhao et al., 2014). The protein sequence can be used to model the structure of the channel and identify amino acids critical to channel function, insecticide binding, conductance ion selectivity, and desensitization (Chen et al., 2006).

Another intereresting set of ligands interacting with the channels of LGIC family members are the avermectin/ivermectin compounds, best known for anti-parasitic nematode toxicity (Pong and Wang, 1982), These macrocyclic microbial molecules bind at a novel site in the nematode GluCl channel (Hibbs and Gouaux, 2011), with capability of opening the channel, an efficacy also found for vertebrate PLGIC such as glycine receptors (Asatryan et al., 2010) and GABAARs (Williams and Yarbrough, 1979; Supavilai and Karobath, 1981b; Drexler and Siegharrt, 1984; Olsen and Snowman, 1985; Olsen et al., 2014).

2.1.4. General Anesthetic Sites—We (Ticku and Olsen, 1978) showed that the barbiturates (allosterically) inhibited picrotoxinin binding, consistent with enhancing $GABA_AR$ currents in neurons, and producing sedative and anticonvulsant efficacy in animals (Bowery and Dray, 1976; Nicoll et al., 1975; Ransom and Barker, 1975). The barbiturates (Leeb-Lundberg et al., 1980; Willow and Johnston, 1981; Olsen and Snowman, 1982) and related CNS depressants like etazolate (Leeb-Lundberg et al., 1981a; Supavilai et al., 1981a) and neuroactive steroids enhance $GABA_AR$ currents and modulate BZ binding to the BZ sites on $GABA_ARs$, as well as the GABA binding site (Willow and Johnston, 1981; Whittle and Turner, 1982; Olsen and Sapp, 1985; Turner et al., 1989) and enhance the GABA enhancement of BZ binding to $GABA_ARs$ (Skolnick et al., 1981). We demonstrated for a large series of barbiturates and related compounds' inhibition of picrotoxin binding in a chemically and stereospecific manner that correlated with their potency as PAMs on $GABA_AR$ currents and as sedative/hypnotic/anesthetics in organisms (Olsen et al., 1986). It eventually became clear that many general anesthetics act, at least in part, by enhancing $GABA_AR$-mediated inhibition in the brain by binding directly to sites on the $GABA_AR$ proteins that produce PAM efficacy and modulation of the various ligand binding sites on $GABA_ARs$ (Johnston, 1984; Peters et al., 1988; Olsen et al., 1991; Mihic et al., 1997; Forman and Chin, 2008). In particular the intravenous anesthetics (etomidate, propofol, barbiturates), and neuroactive steroid anesthetics (Harrison and Simmonds, 1984; Smith, 2003) act in a chemically and stereospecific manner, and also more potently than on other potential brain targets including various ion channels (Sigel and Steinmann, 2012; Olsen and Li, 2011).

Based on the long-known anesthetic efficacy of progesterone (Selye, 1942) and the clinical utilization of its synthetic analogue alphaxalone (Glaxo: 5α-pregnan-3α-ol-11,20- dione) as an intravenous short-acting general anesthetic, certain endogenous neuroactive steroids, such as allopregnanolone (progesterone metabolite) and tetrahydro-deoxycorticosterone (cortisone metabolite), are established specific $GABA_AR$ PAMs (Majewska et al., 1986; Smith et al.,1987; Gee et al., 1988; Turner et al., 1989; Olsen and Sapp, 1995; Smith, 2003; Belelli and Lambert, 2005). The group of steroids active on the nervous system are classified as neuroactive steroids, comprised of endogenous steroids (termed neurosteroids), many of them hormones and hormone metabolites, and synthetic analogues. The physiological roles for neurosteroids as GABAAR PAMs and as links between nervous and endocrine systems are clearly important (Barbaccia et al., 2001; Concas et al., 1998; Maguire et al., 2005; Morrow et al., 1999; Paul and Purdy, 1992; Smith, 2003), but beyond the scope of this review. Binding sites are discussed below.

Given the convincing evidence that $GABA_ARs$ are important targets of general anesthetics, both volatile agents and alcohols, (long chain and high dose ethanol), and i.v. anesthetics: propofol, etomidate, barbiturates, and steroid anesthetics (Franks and Lieb, 1994; Olsen et al., 2004), how can we identify anesthetic binding sites? Site-directed mutagenesis (Mihic et al., 1997), substituted cysteine modification protection (SCAMP) (Forman and Miller, 2016), or photoaffinity labeling (Forman and Miller, 2011; Olsen and Li, 2011) have been successful approaches in this regard.

Site-directed mutagenesis showed certain residues in the TMD of both $GABA_A$Rs and the related glycine receptors (GlyRs) are critical for modulation by volatile anesthetics and long-chain alcohols (Mihic et al., 1997). Two residues, one in TM2 (α1S270 or α2S270, or corresponding β1S265 or β3N265) and one in TM3 (α1A291 or corresponding β1M286) were identified as essential for modulation by the two types of anesthetic. These residues are suggested to line an aqueous pocket on the exterior of the TMD ion channel domain within individual subunits where anesthetic binding can perturb channel gating by GABA. Active volatile and i.v.anesthetics show a 'cut-off' in maximum ligand volume; this depends on size of the amino acids in the putative pocket (Wick et al., 1998; Koltchine et al., 1999; Jenkins et al., 2001; Krasowski et al., 2001). However, it is difficult to explain how ligands of such chemical structural diversity could all bind to the same intrasubunit pocket to modulate GABAAR function. Support for a role of these two critical "Mihic" residues came from independent sources. Belelli et al., (1997) demonstrated that GABAAR sensitivity to etomidate, like its analog loreclezole, showed greater sensitivity for β2 and β3 subunits than for β1, explained by a single residue β2/3N265 in M2, corresponding to one of the "Mihic" residues. The TM2 residue β2/3N265 was shown by mouse knock-in engineering to be critical for in vitro (Siegwart et al., 2002) and in vivo action of etomidate, propofol, and to a much lesser extent also the volatile anesthetics (Jurd et al., 2003; Reynolds et al., 2003). Mutation of these two residues to cysteine and alkylation inhibited anesthetic modulation, and the alkylating blockade could be protected (SCAMP) by including excess free anesthetics, e.g., propofol or etomidate (Bali and Akabas, 2004).

The arrangement of TM1-2-3 in the membrane could be modeled in a manner consistent with agonist- and modulator-sensitive conformational changes. Mutations of the residues was shown to reduce anesthetic actions by volatile and long-chain alcohol anesthetics in vivo (Werner et al., 2011), but EtOH was active only at ≥100 mM (Lobo and Harris, 2008); they argued that, although not demonstrated, EtOH might act on some $GABA_A$R subtypes at <100 mM (see below). Importantly, mutation of GABAAR β265 to cysteine allowed covalent attachment by an alkylating analogue of n-propanol with resulting persistent channel enhancement (Mascia et al., 2000), suggesting that propanol and EtOH could bind to this site at pharmacologically relevant intoxicating concentrations.

Based on site-directed mutagenesis alone, our lab (Carlson et al., 2000; Chang et al., 2003) demonstrated that mutating residues in anesthetic-sensitive GABAAR β subunits into those of anesthetic-insensitive rho subunits in the Pre-M1/M1 region reduced sensitivity to numerous general anesthetics in binding and in vitro functional assays when mutating one single glycine residue situated near the top of the M1 channel gate. This residue has not been studied further due to the development of other approaches. Likewise, mutagenesis of the TMD regions demonstrated four amino acids needed for neuroactive steroid modulation of GABAAR channels allowing binding sites to be proposed, based on an homology model derived from the *Torpedo* nAChR (Unwin, 2005). One site with two residues in the TMD, α1M236 and βY284, initiates direct activation, whereas a second site with αQ241 and αN407 mediates the potentiation of responses to GABA. These ligands have the highest efficacy on $GABA_A$Rs containing δ or α5 subunits (Hosie et al., 2006). These possible sites are considered further below.

TM3 residue βM286 was identified by affinity labeling with an azi-etomidate reagent as a binding site for etomidate (Li et al., 2006). We, a team headed by Keith Miller including our lab, used the etomidate analog [^{3}H]azietomidate and brain heteromeric receptors, not recombinant models, for the first successful identification of anesthetic binding sites using photolabeling. We identified binding to $GABA_AR$ at the β+/α− interface (α1M236 in TM1and β3M268 in TM3: Li et al., 2006). These sites are thus intersubunit, not intrasubunit, like those for GABA, and seemingly more efficient and logical. The volatile anesthetic binding site residues in α or β subunits TM3 and β subunits TM2 (Mihic et al. 1997) seems to be the same as (TM3), or, close but not overlapping (TM2), with our proposed inter-subunit binding site(s) for the intravenous general anesthetics etomidate and propofol (Li et al., 2010; Rudolph and Antkowiak, 2004). Li et al.,(2009) showed that the GABAAR-modulatory neuroactive steroids enhanced, rather than blocking the azi-etomidate labeling, indicating activity via another binding site; the residues proposed by Hosie et al., (2006), although close to the inter-subunit etomidate binding residues, were not identical and in fact were not close to the sites modeled by helical arrays of the TMD regions. Of course the protein could have more flexibility than the models suggested by crystal structures. Li et al. (2010) found that volatile anesthetics inhibited the binding of azi-etomidate, long chain alcohols did not; propofol and active barbiturates inhibited partially.

The Keith Miller team went on to develop affinity labels based on propofol and barbiturates and identify binding residues by sequencing. The mephobarbital analog, more potent as an anesthetic and enhancer of GABAAR than the parent mephobarbital, labeled the γ+/β− and α+/β− interfaces (2/5 available), and binding was inhibited by cold barbiturate and propofol, but not etomidate nor the steroid anesthetic alphaxalone (Chiara et al., 2013). This is internally consistent but does not explain how barbiturates inhibited etomidate binding (Li et al., 2010). It could be that the two sites are allosterically coupled, or the native brain protein used by Li et al., (2010) could include multiple receptor subtypes with differing potency selectivities. Maldifassi et al. (2016a) suggest predominant binding of barbiturates to the αβ +/α−γ interface and in addition to the α+/β− and/or α+/γ− interfaces in α1β2γ2.

An anesthetic azi-propofol analog was created by the Keith Miller team and used to identify binding residues in recombinant heteromeric GABAARs. Jayakar et al., (2014) identified labeling at the β+/α−, γ+/β−, and α+/β− interfaces, that is all four of the sites labeled by azietomidate (β+/α−, 2 copies), and the barbiturate photolabel (γ+/β− and α+/β−). None of the three ligands bound to the α+γ/− interface. So barbiturates and etomidate act at homologous but different sites on the same proteins, while propofol acts on all the sites the other two act on. I think pentobarbital acts weakly on the same sites as etomidate in addition to its own sites (Li et al., 2010). Subunit concatenations with or without β2N265I mutation suggest propofol binds predominantly on the γβ+/α−β and γ+/β− interfaces on the α1β2γ2 $GABA_AR$ (Maldifassi et al., 2016a). Another propofol affinity label analog was developed (Yip et al., 2013) and found by mass spectrometry to bind to a residue b3H267 near the top (extracellular) end of M2, far from the sites found by Jayakar et al. (2014); this binding was not demonstrated to be inhibited by excess non-modified propofol.

This discovery of anesthetic binding sites at subunit interfaces, plus the pharmacological distinctions for different chemical classes of ligands at homologous pockets in the five

interfaces, seems to be a major insight into the multiple but related types of anesthetic mechanism unearthed by the affinity labeling approach of the Keith Miller team (Forman and Miller, 2016).To quote from that paper, “further research is needed to establish the relative affinities and efficacies of these sites and their dependence on subunit composition”. The steroid anesthetics do not inhibit the binding of any of the three classes of sites identified by photolabeling (etomidate, profpofol, barbiturates), and the site has not been identified. A successful steroid anesthetic affinity labeling was achieved, showing stereo-selective covalent attachment of an azi-steroid to F301, identified by mass spectrometry, in the TM3 domain of recombinant homomeric β3-$GABA_AR$ (Chen et al., 2012). Another remaining question is the role of the M2 βN265 residue that is clearly required for the activity of many anesthetics (Mihic et al., 1997; Belelli et al., 1997; Jurd et al., 2003). How can it bind all the difference classes of anesthetic ligands? The SCAMP data are not compelling for a competitive block by the anesthetic ligands (Forman and Miller, 2016) so perhaps it is allosterically coupled to all the ligand binding sites. Photolabeling studies also suggest binding sites for etomidate and barbiturates at α4β3δ and α4β3 $GABA_AR$ subtypes at the β+/α−, and (“most likely”) at the β+/β− interfaces, respectively, which were not binding alphaxalone or DS2 (Chiara et al., 2016). Table 1 summarizes this.

Interestingly, the homomeric prokaryotic PLGIC protein ELIC, a structural model of GABAARs, was found to be activated by GABA and modulated by benzodiazepines (Spurny et al., 2012), suggesting strongly that these simpler versions of PGLICs can provide valuable information about native PLGICs including GABAARs. Earlier, the homologous homomeric prokaryotic PLGIC protein GLIC was shown to bind general anesthetics propofol and desflurane in crystal structure, and these ligands acted as NAMs (not PAMs) on the channel flux (Nury et al., 2011; reviewed in Sieghart, 2015). Further, Bromstrup et al., (2013) showed that point mutations in TM2 could convert GLIC binding of general anesthetics from intrasubunit to intersubunit sites, while also converting the ligands from NAMs to PAMs. Since anesthetics are PAMs in native GABAARs, the slightly mutated GLIC becomes an even better model of native anesthetic-sensitive eukaryotic PLGICs. Hopefully, remaining murky areas will be quickly resolved (Howard et al. 2014).

To summarize, specific binding sites for these anesthetics (Olsen et al., 2004) have been identified by photoaffinity labeling at subunit interfaces in the transmembrane domain (see Fig. 1) of $GABA_AR$ proteins (Li et al., 2006; Olsen, 2015; Forman and Miller, 2016). The essential amino acids have been verified by in vivo anesthesia testing. Genetically engineered mice demonstrated that general anesthetic action not only requires $GABA_ARs$, but also requires specific subtypes of $GABA_ARs$ in specific brain locations (Jurd et al., 2003; Reynolds et al., 2003; Rudolph and Antkowiak, 2004); this appears to result from slight differences in the ligand binding sites in $GABA_AR$ subtypes (Chiara et al., 2013), reviewed in Table1 (Olsen, 2015).

2.1.5. High Affinity Ethanol Site—In addition, our lab has demonstrated that certain subtypes of $GABA_AR$, involved in tonic inhibitory currents via extrasynaptically localized membrane sites, are involved in low millimolar PAM action on $GABA_ARs$ by EtOH (Wallner et al., 2003; Hanchar et al., 2005; Hanchar et al., 2006; Wallner et al., 2006b). EtOH appears to bind to a modified GABA/BZ binding site in the ECD of δ subunit-

containing $GABA_A$Rs (Olsen et al., 2014; Wallner et al., 2014). These workers proposed tonic current-carrying $GABA_A$Rs as low dose EtOH targets.

Many years of evidence support a role for $GABA_A$Rs in mediating the anxiolytic, mood-enhancing alcohol effects experienced by humans with blood alcohol levels usually below the US 17 mM legal driving limit (Suzdak et al., 1986; Aguayo et al., 2002; Kumar et al., 2004; Wallner et al., 2006a; Olsen et al., 2007). In vitro, the function of many proteins, including $GABA_A$Rs (Mihic et al., 1997) are modified at high >50 mM ethanol (EtOH) concentrations, which might be regarded as relevant to toxicology but of debatable relevance to intoxication. A few labs have reported direct enhancement of GABA-stimulated $^{36}Cl^-$ flux in brain homogenates (Suzdak et al., 1986; Morrow et al., 1988; Leidenheimer and Harris, 1992) but the electrophysiologists did not consider this convincing, nor was it generally reproduced. However, essentially consensus exists that tonic GABA inhibitory Cl^- current-dependent transmission is uniquely sensitive to alcohol at relevant low (≤30 mM) EtOH concentrations (Roberto et al., 2003; Wei et al., 2004; Carta et al., 2004; Hanchar et al., 2005; Liang et al., 2006; Jia et al., 2007; Mody et al., 2007; Santhakumar et al., 2007; Jia et al., 2008; Santhakumar et al., 2013; Fleming et al., 2013; Herman et al., 2013; Herman and Roberto, 2016; Centanni et al., 2017). Some workers have suggested that this enhancement by EtOH involves presynaptic effects with increased GABA neuron activity and GABA release (Siggins et al., 2005; Weiner and Valenzuela, 2006; Breese et al., 2006), but more likely there is in addition direct enhancement of GABAAR tonic currents on sensitive cells (reviewed by Wallner et al., 2006a). Further, very high EtOH sensitivity (≤10 mM) has also been reported in recombinantly expressed (extrasynaptic δ subtype) α4/6βδ receptors (Sundstrom-Poromaa et al., 2002), with significant β3 selectivity (Wallner et al., 2003, Wallner et al., 2014); this observation also has been challenged (Borghese et al., 2006). The positive results and possible difficulties in detection have been reviewed (Wallner et al. 2006a; Olsen et al. 2007).

At least four lines of evidence support a role of a homologous BZ-like site in the ECD of certain GABAAR subtypes that mediates low dose EtOH actions (Wallner et al. (2017). I paraphrase from that article: First, an influential BZ-binding site residues at the α+/γ− interface (histidine H100) present in ‘diazepam-sensitive’ α1,2,3,5 GABAAR subunits, is instead arginine (R) in ‘diazepam-insensitive’ α4,6 GABAAR subunits. The α6R100Q mutation is a naturally-occurring polymorphism initially found in Alcohol Non-Tolerant (ANT) rats that show increased benzodiazepine AND alcohol-induced motor impairment (Korpi et al., 1993). That paper showed in vitro sensitivity of the α6R100Q mutation but no enhancement of wild type or mutant receptors by EtOH. We showed α6R100Q leads to increased EtOH sensitivity when recombinantly expressed with β3 (but not with β1 or β2) *and δ* subunits, as well as increased EtOH effects on tonic currents in cerebellar slices of the mutant rat at concentrations as low as 10 mM, and leads to increased EtOH-induced motor incoordination in rats homozygous for the α6R100Q mutation (Hanchar et al., 2005). The α6R100Q polymorphism was also found to be enriched in two independently generated alcohol behavioral (non-EtOH-preferring) rat lines (Saba et al., 2001; Carr et al., 2003).

Second, the (imidazo)benzodiazepine Ro15-4513 (a close structural analog of the BZ antagonist flumazenil), reported as a behavioral alcohol antagonist in rats that specifically

blocks GABAAR-mediated Cl^- flux (Suzdak et al., 1986), also reverses low concentration EtOH enhancement of α4/6β3δ receptors in oocytes using voltage clamp recordings (Wallner et al., 2006b) in a competitive manner. [^{3}H]Ro15-4513 binds to α4/6β3δ receptors but not to α4/6β3γ2 receptors in a manner inhibited by EtOH (10-20 mM) (Hanchar et al., 2006). Our data suggest that the in vivo block of some alcohol behaviors in some animals in some labs (Suzdak et al., 1986; Lovinger and Homanics, 2007) can be explained by the in vitro block by Ro15-4513 of the EtOH-sensitive δ subunit-containing GABAAR subtypes. Chemical analogues of imidazo-benzodiazepines and beta-carbolines show pharmacological specificity for the [^{3}H]Ro15-4513 binding sites and to inhibit EtOH enhancement ofα4/6β3δ receptors (Wallner and Olsen, 2008). Third, higher alcohol sensitivity of α4/6β3δ $GABA_A$Rs (compared to other β subunits: Wallner et al., 2003) is due to a single amino acid difference in the three mammalian β subunits; β1S66, β2A66, β3Y66 (Wallner et al., 2014). Position 66 in $GABA_A$R beta subunits is homologous to position γ2A79 that lines the classical BZ α+/γ− binding site (Kucken et al., 2003). These findings led us to a model where the EtOH and overlapping Ro15-4513 site is located at α4,6+/β3− interfaces in ECD of δ-$GABA_A$Rs (Wallner et al., 2014).

A tentative structural model of the $GABA_A$R protein showing the binding sites for the major classes of ligands is shown in Fig. 1, both the extracellular domain (ECD) sites for GABA, BZ, and EtOH, and the transmembrane domain (TMD) showing the sites for anesthetics and channel blockers, while Table 1 summarizes the various categories of PAMs with binding sites on GABAARs and their locations (Olsen, 2015).

Additional candidate drugs for alcohol antagonism have been described for natural products, especially herbal medicines. A major candidate group are the flavonoids, found in numerous plants with reported positive pharmacological effects, including anxiolysis (Hanrahan et al., 2015), some BZ-like agonists (Kahnberg et al., 2002). One such flavonoid, dihydromyricetin, was shown to block EtOH actions in vitro on GABAAR and in vivo, in a flumazenil-sensitive manner, suggesting a GABA connection (Shen et al., 2012).

Fourth, evidence for an important role of GABAAR in acute and chronic effects of EtOH use and abuse are summarized in reviews (Olsen and Spigelman, 2012; Olsen and Liang, 2017). Using the chronic intermittent ethanol (CIE) rodent model of alcohol dependence (Kokka et al., 1993), we have demonstrated that moderate to high doses of EtOH cause a rapid within minutes down-regulation of the early responder GABAAR α4/6βδ subtypes mediating tonic inhibition (Cagetti et al., 2003; Liang et al., 2007; Gonzalez et al., 2012), followed within hours by down-regulation of synaptic α1βγ2 subtypes and up-regulation of α4βγ2 with increased synaptic localization and increased BZ-insensitive miniature inhibitory synaptic potentials (mIPSCs) (Liang et al., 2007), and up-regulated synaptic α2βγ GABAAR subtypes mediating EtOH-sensitive mIPSCs (Lindemeyer et al., 2017). The plastic changes in GABAAR are all transient but can be related to numerous behavioral alterations related to tolerance, withdrawal, and development of alcohol dependence; all the changes become persistent following a critical number of cycles of EtOH-induced CNS depression followed by mini-withdrawal rebound hyperexcitability (Kokka et al., 1993; Liang et al., 2006). We have argued (Lindemeyer et al., 2017) that the up-regulated synaptic α2βγ GABAAR subtypes mediating EtOH-sensitive mIPSCs are strong candidates for

contributing to the maintained anxiolytic action of EtOH in limbic system circuits, while also contributing to the dopamine-dependent reward circuitry activation via a mechanism of presynaptic inhibition onto GABAergic neurons that inhibit dopamine neurons.

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Highlights

- Many clinical CNS depressants act by enhancing $GABA_A$ receptor-mediated inhibition (83)
- Some anxiolytic and sedative drugs act on $GABA_AR$ subtype-dependent ECD sites (78)
- General anesthetics, alcohols, steroids act at $GABA_AR$ subunit-interface TMD sites (83)
- Ethanol at high anesthetic doses acts on $GABA_AR$ subtype-dependent TMD sites (77)
- Ethanol at low intoxicating doses acts at $GABA_AR$ subtype-dependent ECD sites (79)

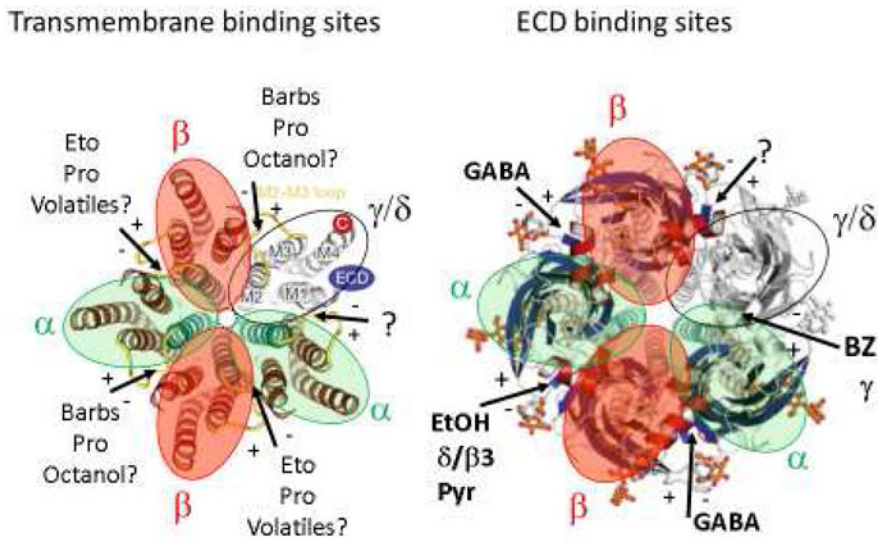

FIGURE 1.

GABAAR ligand sites at subunit interfaces identified by mutagenesis and/or affinity labeling. The left panel shows the transmembrane binding sites, while the right panel shows the ECD binding sites. The protein structures are taken from the X-ray crystallography-derived structure of the recombinant mammalian homomeric β3 $GABA_AR$ (Miller and Aricescu, 2014), on which is displayed an homologous native $GABA_AR$ comprised of an α–β–α–β–γ/δ heteropentamer (actual subunits arbitrary, no specific sequence implied although they are all homologous to β3). The protein is viewed looking from the extracellular face, perpendicular to the cell membrane/synapse. Thus the ECD pentamer on right would actually be positioned directly on top of the TMD pentamer at left. Both portions indicate locations of ligand binding sites found in the β3 homomer structure. The two α subunits are indicated by the green (or dark gray) shaded oval, the two β subunits by the pink (or light gray) shaded ovals, and the one γ/δ subunit indicated by the clear (white) oval. An example C-terminus is indicated by a small red circled "C" at the bottom of the TMD of the γ/δ subunit; the M1,2,3,4 domains are also labeled in this example subunit, and the N-terminus of the TMD of each subunit would attach to its ECD at the position indicated by the small blue (or black) oval "ECD". Ligand binding sites for the compounds listed (in shorthand) are indicated by arrows (Cf. Table 1). Note that the heteropentameric proteins show several different but homologous subunit interfaces (Table 1) so the pharmacological specificity varies with GABAAR subtype. The ligands named are BZ (benzodiazepines) and GABA, EtOH, and Pyr (pyrazoloquinoliines, see Table 1) in the ECD. In the TMD, Eto (etomidate), Pro (propofol), octanol, volatiles (volatile anesthetics), and barbs (barbiturates) binding sites are located.

Table 1

Summary of GABAAR ligand sites at subunit interfaces identified by mutagenesis and/or a **a**ffinity labeling. Cf. Figure 1.

Interface /	Extracellular Domain	Trans-membrane Domain
β+/α−	GABA [1,2]	Etomidate [3-6]
(2 copies/pentamer)		Propofol [3,6-10]*
		Volatiles anesthetics? [4,10]
α+/β−	EtOH (on δ) [11]	Barbiturates [12]
	Imidazo-BZ (on δ) [11]	Propofol [9]
	Pyrazoloquinolines [13]	Octanol ? [9]
γ+/β−	?	Barbiturates [12]
		Propofol [9]
		Octanol? [9]
α+/γ−	BZ [14,15,16]	?
α+/δ−	?	?
δ+/β−	?	Barbiturates? [8]
		Propofol? [8]
		Octanol? [7]
?		Neuroactive steroids [17]#,^

1: Amin & Weiss, 1993;

2: Smith & Olsen, 1994;

3: Li et al., 2006;

4: Mihic et al., 1997;

5 Belelli et al., 1997;

6; Jurd et al., 2003;

7: Bali & Akabas, 2004;

8 Krasowski et al., 2001;

9: Jayakar et al., 2014;

10: Li et al., 2010;

11: Wallner et al.,2014;

12: Chiara et al., 2013;

13 Ramerstorfer, et al., 2011;

14: Duncalfe et al., 1996;

15 Smith & Olsen, 1995;

[16] Sawyer et al., 2002;

[17] Li et al., 2009.

[*] A residue in the TMD (M3) but not at subunit interfaces was affinity labeled by a propofol analog (Yip et al., 2013);

[#] Four residues in TMD (TM 1,2,3,4) were identified as critical for neuroactive steroid action (Hosie et al., 2006);

[^] A different residue in the TMD (M3) but not at subunit interfaces was affinity labeled by a neuroactive steroid (Chen et al., 2012).

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