GABA receptors: pharmacological potential and pitfalls

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Abstract

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, plays a key role in the regulation of neuronal transmission throughout the brain, affecting numerous physiological and psychological processes. Changes in GABA levels provoke disbalance between excitatory and inhibitory signals, and are involved in the development of numerous neuropsychiatric disorders. GABA exerts its effects via ionotropic (GABA_A) and metabotropic (GABA_B) receptors. Both types of receptors are targeted by many clinically important drugs that affect GABAergic function and are widely used in the treatment of anxiety disorder, epilepsy, insomnia, spasticity, aggressive behaviour, and other pathophysiological conditions and diseases. Of particular importance are drugs that modulate GABA_A receptor complex, such as benzodiazepines, barbiturates, neuroactive steroids, intravenous and inhalational anesthetics, and ethanol. Molecular interactions and subsequent pharmacological effects induced by drugs acting at GABA_A receptors are extremely complex due to structural heterogeneity of GABA_A receptors and existence of numerous allosterically interconnected binding sites and various chemically distinct ligands that are able to bind to them. There is a growing interest in the development and application of subtype-selective drugs that will achieve specific therapeutic benefits without undesirable side effects. The aim of this review is to briefly summarize the key pharmacological properties of GABA receptors, and to present selected novel findings with the potential to open new perspectives in the development of more effective therapeutic strategies.

Keywords: GABA, GABA receptor pharmacology, benzodiazepines, neurosteroids
Introduction to GABA receptor diversity and pharmacological complexity

As the principal mediator of inhibitory synaptic transmission in the mammalian brain, GABA exerts its effects and shapes behaviour via two different types of GABA receptors. While fast ionotropic GABA_A receptors belong to the family of ligand gated ion channels, metabotropic GABA_B receptors are receptors with seven transmembrane domains coupled to G-proteins. Activation of GABA receptors by GABA induces membrane hyperpolarization, reduces the frequency of the generation of action potentials, and results in inhibition. Mainly local-circuit interneurons that constitute 15-20% of all cortical neurons predominantly use GABA as the neurotransmitter [1-3].

Most of its effects GABA exerts via GABA_A receptors. GABA_A receptors may be embedded in postsynaptic membrane where they mediate transient and fast synaptic inhibition that occurs in milliseconds, or may be located at the extrasynaptic places where respond to ambient concentrations of GABA and mediate long-term inhibition. In fast synaptic inhibition, GABA released from the presynaptic membrane terminals rapidly diffuses across the synaptic cleft and binds to binding sites at postsynaptic GABA_A receptors. Following binding, these receptors undergo a rapid conformational change that opens integral chloride channel and permit the flow of chloride ions down their chemical gradient, across the postsynaptic membrane. This ensures propagation of neurotransmission and represents basis of neural communication [4,5].

In contrast to GABA that directly activates and opens ion channels, some drugs, such as competitive antagonist bicuculline, selectively block GABA_A receptors and prevent inhibitory action of GABA. Muscimol, the principal psychoactive constituent of the mushroom Amanita muscaria, also acts as a potent, selective agonist of GABA_A receptors. A variety of clinically important drugs, including anticonvulsants, anxiolytics, general anesthetics, barbiturates, ethanol and neuroactive steroids, as well as drugs important as research tool such as convulsive picrotoxin, allosterically modulate GABA-induced activation of GABA_A receptors by acting on distinct binding sites on the receptor and modulate GABA action [5,6]. These drug interactions, together with structural diversity of GABA_A receptors, shape the complex nature of GABA_A receptor pharmacology, although simultaneously offer a great opportunity for the design of novel and more selective compounds for improved therapeutic applications [7]. Unfortunately, severe problems are related to the long-term use of drugs acting at GABA_A receptors, including loss of efficacy, and development of tolerance.
and dependence that largely limit the time window of their desirable therapeutic benefits [4,8-11].

GABA also achieves inhibitory effects by acting at G-protein-coupled metabotropic GABA_{B} receptors. The slow, longer-lasting inhibition of GABA_{B} receptors is mediated by indirect gating of either potassium or calcium channels via second messengers. Characteristic agonists of GABA_{B} are the GABA analog baclofen and 3-aminopropylphosponous acid (3-APPA; CGP27492), while saclofen, phaclofen and 2-hydroxysaclofen act as antagonists of GABA_{B} receptors [12].

Old nomenclature also included GABA_{C} receptors that are expressed predominantly in retina. However, International Union of Basic and Clinical Pharmacology has recommended that GABA_{C} receptors should be assumed as one of the many isoforms of GABA_{A} receptors [5].

**GABA_{A} receptors and their subunits**

Ubiquitously expressed GABA_{A} receptors are the major receptors of inhibitory neurotransmission across the central nervous system (CNS) that play a major role in virtually all brain physiological functions. GABA_{A} receptors are heteropentameric ligand-gated proteins that form a channel specific for chloride ions as an integral part. GABA_{A} receptors are members of the Cys-loop ligand-gated ion channel superfamily due to the presence of a disulphide-bridged loop in the extracellular domain. They share structural and functional homology with other members of the family. In addition to GABA_{A} receptors, the main pentameric receptors within the Cys-loop superfamily are nicotinic acetylcholine (nACh), 5-hydroxytryptamine type 3 (5HT_{3}) and glycine receptors [13,14].

GABA_{A} receptors are assembled from an impressive repertoire of different homologous subunits that are encoded from the pool of 19 different genes. Six α (α_{1-6}), three β (β_{1-3}), three γ (γ_{1-3}), δ, ε, θ, π and three ρ (ρ_{1-3}) subunits have been identified by molecular cloning techniques [6,14-16]. In some cells expression of more than eight different isoforms has been demonstrated [5]. Throughout the CNS some subunits are broadly expressed, while others exhibit more restricted expression profile [17,18]. For example, the most abundant α_{1} subunit is expressed almost ubiquitously in the brain, α_{5} is highly expressed in the hippocampus, α_{6} subunit is expressed only in granule cells of the cerebellum, while ρ-subunits, the major molecular components of the formerly called GABA_{C} receptors, are expressed mainly in the retina [19-22]. Expression of β_{2} subunit strongly correlates with that
of α1, while expression pattern of β3 subunit resembles to that of α2. Isoform γ2 is the most widely distributed isoform of γ subunit [17].

GABA\(_A\) receptor subtypes composed of different subunits represent distinct receptor populations that exhibit unique functional and pharmacological properties, and are differentially regulated at the transcriptional, post-transcriptional and translational levels [23]. GABA\(_A\) receptor subtypes are expressed in a specific spatio-temporal pattern in the developing and adult CNS. Some combinations of subunits, such as α1β2γ2, the most prevalent adult isoform, are broadly distributed in different brain areas, while the expression of some other subtypes of GABA\(_A\) receptors is restricted to certain regions [24].

Mature subunits share a common topological organization, and have approximately 450 amino acid residues in length. Subunits consist of a large extracellular N-terminal domain that contains already mentioned Cys loop, followed by four transmembrane domains (TM1-TM4), and a short extracellular C-terminal domain. TM2 domains of all five subunits line the lumen of ion channel. There is a large phosphorylatable intracellular loop between TM3 and TM4 domains of each subunit that participates in protein-protein interactions and plays an important role in the regulation of GABA\(_A\) receptor function. Some of the intracellular proteins that interact with GABA\(_A\) receptors are GABA\(_A\) receptor associated protein (GABARAP), Golgi-specific DHHC zinc finger protein (GODZ) phospholipase C-related, catalytically inactive proteins PRIP-1 and PRIP-2, Plic-1, radixin, GABA\(_A\) receptors interacting factor-1 (GRIF-1), and brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2). These proteins interact with particular subunits at GABA\(_A\) receptor complex and modulate broad spectrum of GABA\(_A\) receptors activities, such as trafficking enhancement, surface stabilization, receptor internalization, anchoring of receptors in the cytoskeleton, and regulation of modification of GABA\(_A\) receptors [5,25].

Chromosomal mapping techniques revealed that the majority of genes coding for the subunits of GABA\(_A\) receptors are localized into four clusters on chromosomes 4, 5, 15 and X in the human genome, and within these clusters genes have conserved transcriptional orientation [26]. Additional diversity of receptor structure is accomplished by alternative splicing of some of the subunit mRNAs and by RNA editing that might affect kinetics, subunit assembly and cell-surface expression of GABA\(_A\) receptors [27]. For example, the γ2 subunit appears in three alternatively spliced variants termed γ2L, γ2S and γ2XL [28], although physiological and pharmacological relevance of this diversity is still not completely resolved. Nova, a neuron-specific RNA binding protein, regulates alternative splicing of γ2 subunit by acting on an intronic splicing enhancer which lies far downstream of the regulated
exon, and general dysregulation of Nova's splicing enhancer function may underlie the neurologic defects seen in patients with Nova's absence [29].

Only a limited number of mutations have been found in GABA<sub>4</sub> receptor subunit genes that are specific for different diseases. For example, point mutations in the α1 and γ2 subunits are found in patients with genetic epilepsies, while single nucleotide polymorphisms (SNPs) in the gene encoding the α2 subunit are involved in alcohol dependence and illicit drug dependence. The gene encoding the β1 subunit also has been linked to alcohol dependence and to bipolar disorder. The genes encoding the α1, α6, β2 and π subunits have been linked to schizophrenia [4].

**Subunit composition determines pharmacological effects**

It is still not resolved which subunits assemble together to form different isoforms of GABA<sub>4</sub> receptors that really exist in nature [14]. Numerous expression studies using recombinant receptors indicated that receptors composed of α, β, and γ subunits form GABA<sub>4</sub> receptors with a pharmacological profile that mimic properties of the majority of native receptors. Despite the fact that the immense number of different pentameric combinations can be assembled from the pool of native protein subunits, it seems that the prevalent form of native GABA<sub>4</sub> receptors is the combination of two α, two β and a single γ, δ or ε subunit [30,31]. As mentioned before, isoform α1β2γ2 is considered as the most prevalent subtype of adult GABA<sub>4</sub> receptors, with γ2β2α1β2α1 counter-clockwise arrangement surrounding central ion pore when viewed from the synaptic cleft [32]. It is estimated that approximately 60% of all GABA<sub>4</sub> receptors have the subunit composition α1β2γ2, approximately 15–20% have the α2β3γ2 combination, approximately 10–15% have the α3βnγ2 combination, approximately 5% have the α4βnγ or α4βnδ combination, less than 5% have the α5β2γ2 combination and, likewise, less than 5% have the α6β2/3γ2 combination [4]. Furthermore, it is possible for GABA<sub>4</sub> receptors to contain two different α subunits. Thus, α1α6βxγ2 and α1α6βxδ receptors have been found in the cerebellum [33].

Composition and arrangement of GABA<sub>4</sub> receptors subunits shapes the synaptic response to GABA, as well as electrophysiological, functional and pharmacological properties of GABA<sub>4</sub> receptors, including potency and efficacy of different drugs to exert their effects [6,34,35]. For example, Zezula et al. [36] systematically investigated properties of recombinant GABA<sub>4</sub> receptors formed after transfection of human embryonic kidney (HEK) 293 cells with α1-, β3-, or γ2-subunits, or with various combinations of these subunits. From
all possible subunit combinations, high affinity binding sites for GABA were formed in cells transfected with α1β3- or α1β3γ2-subunits only. Binding of benzodiazepines were induced in cells after transfection with α1γ2- or α1β3γ2 subunits, whereas binding sites for convulsant [35S]t-butylbicyclophosphorothionate (TBPS) were found in cells transfected with β3-, β3γ2-, α1β3-, or α1β3γ2-subunits. Binding of [35S]TBPS was inhibited by pentobarbital (barbiturate), alphaxalone (anesthetic neuroactive steroid) and propofol (anesthetic) with a potency which differed in cells transfected with β3-, β3γ2-, α1β3-, or α1β3γ2-subunits, demonstrating that receptors with different subunit composition exhibit distinct pharmacological properties.

**Tonic and phasic inhibition by GABA\textsubscript{A} receptors**

Some subunits, such as δ subunits, are placed only at extrasynaptic places where GABA\textsubscript{A} receptors are activated by low ambient GABA concentrations present in the extracellular fluid, and mediate a so-called tonic inhibition that is relatively non-desensitizing. Isoforms containing γ subunit are expressed predominantly at synapses and mediate rapidly desensitizing phasic GABAergic neurotransmission. Synaptic GABA\textsubscript{A} receptors are activated transiently after the release of GABA from presynaptic vesicles, while extrasynaptic GABA\textsubscript{A} receptors are in general activated continuously, controlling neuronal excitability and the strength of synaptic transmission [37-41].

Tonic and phasic conductances underlie different physiological and behavioural processes [4]. Receptors containing δ subunit regulate numerous behavioural functions, including memory, nociception and anxiety, and may modulate neurogenesis. Taking into account that they are highly responsive to sedative-hypnotic and sleep-promoting drugs, general anesthetics, alcohol and neuroactive steroids, they are considered as potential therapeutic targets for the treatment of memory impairment, insomnia, mood disorders, pain, and post-stroke recovery [39,41-44].

Furthermore, extrasynaptic GABA\textsubscript{A} receptors with δ subunit exhibit a pronounced variability in expression levels under different physiological and pathophysiological conditions that might reshape behaviour and change neuronal susceptibility to different drugs [41]. Modulation of tonic inhibition might be of clinical relevance as extrasynaptic GABA\textsubscript{A} receptors may diminish seizure susceptibility and states of anxiety during the estrous cycle. In particular, in late diestrus in mice (high-progesteron phase), increased expression of δ-containing receptors increases tonic inhibition, suggesting that consequent reduction of
neuronal excitability might be beneficial in women with catamenial epilepsy and premenstrual dysphoric disorder [45]. The large increase in progesterone-derived neurosteroids during pregnancy, and their sharp decline at parturition, may also have considerable effects on GABA<sub>A</sub> receptors during pregnancy and postpartum. Both tonic and phasic inhibition decrease in pregnant mice due to down-regulation of δ and γ2 subunits, and are rebound immediately after delivery. Mice which do not exhibit δ-subunit regulation throughout pregnancy show depression-like and abnormal maternal behaviours [46]. On the other hand, expression of GABA<sub>A</sub> receptors containing δ subunit is diminished in depression and schizophrenia [47,48].

**Endogenous ligands of GABA<sub>A</sub> receptors**

In addition to GABA, neurosteroids are the most important endogenous ligands of GABA<sub>A</sub> receptors. Certain neurosteroids potently and specifically enhance function of GABA<sub>A</sub> receptors, and consequently exert anxiolytic, analgesic, anticonvulsant, sedative, hypnotic and anesthetic properties, and contribute to behavioural effects of psychoactive substances [49]. In the CNS, steroids can be synthesized *de novo* from cholesterol, during stress, pregnancy and after ethanol consumption, and these steroids are called neurosteroids, or can be converted from steroid precursors from periphery [50-52]. *De novo* synthesized neuroactive steroids locally modify neuronal activity in a paracrine manner, and can influence mood and behaviour in various physiological and pathophysiological conditions. GABA-modulatory actions of the pregnane steroids are highly selective, with their actions being brain region- and neuron-dependent. Furthermore, the sensitivity of GABA<sub>A</sub> receptors to neurosteroids is not static but can dynamically change. The molecular mechanisms determining this neuronal specificity include different GABA<sub>A</sub> receptor isoforms, protein phosphorylation and local steroid metabolism and synthesis [53,54].

Relatively recently, it has been discovered that endocannabinoid 2-arachidonoyl glycerol (2-AG) potentiates GABA<sub>A</sub> receptors at low concentrations of GABA, specifically in receptors containing β2 subunit [55]. 2-AG allosterically potentiates GABA<sub>A</sub> receptors via a binding site located in transmembrane segment TM4 of the β2 subunit GABA<sub>A</sub> receptors [56]. The discovery of a direct action of the 2-AG on the β2-containing GABA<sub>A</sub> receptors offers the potential for selective pharmacological intervention that would reduce motility and possibly affect sedation and state of anxiety [56]. Furthermore, it is shown that 2-AG acts synergistically with the neurosteroid tetrahydrodeoxycorticosterone (THDOC; 3α,21-
dihydroxy-5α-pregn-20-one) and modulates extrasynaptic receptors that respond to neurosteroids [55].

**Modulation of GABA_A receptors by pharmacological agents**

GABA_A receptors are targeted by many clinically important drugs. Namely, besides binding site(s) for GABA, GABA_A receptors possess binding sites for a variety of clinically important substances, such as benzodiazepines and benzodiazepine-site ligands, barbiturates, intravenous and volatile anesthetics, ethanol and neuroactive steroids, all of which achieve at least some of their pharmacological effects after binding to GABA_A receptor complex [6,17,57]. These drugs modulate effects of GABA and affect GABAergic transmission, exerting a wide spectrum of pharmacological effects and inducing numerous conformational states of GABA_A receptors.

Diverse approaches have been applied in last years to more precisely identify various binding sites on GABA_A receptor complex, as this knowledge is prerequisite for the design of subtype specific, selective drugs of clinical relevance. Study performed on the comparative models of the extracellular and transmembrane domains of GABA_A receptors in the agonist-free state has revealed that a loose packing of these domains results in a large amount of solvent-accessible space, probably offering a potential explanation for such complex pharmacology and great flexibility of GABA_A receptors [58].

Drugs acting at benzodiazepine binding sites can only modulate GABA_A receptors, while barbiturates, neuroactive steroids and anesthetics at higher concentrations are able to directly activate GABA_A receptors in the absence of GABA [31,59-63]. Binding sites for all of these drugs are allosterically coupled. Therefore, drug that fits or binds to any of these multiple binding pockets induces conformational changes of the receptor complex and modulates GABA-elicited response [64,65]. For some of these multiple binding sites ligands have been identified, whereas for others sites, ligands are unknown. Similarly, the sites of action of large number of compounds that allosterically modulate GABA_A receptors are still not identified [66].

Increased activity of GABA_A receptor activity leads to anxiolysis, ataxia, myorelaxation, sedation, hypnosis, anesthesia and anterograde amnesia, whereas decrease in GABA_A receptor activity leads to increased vigilance, memory enhancement, anxiety and seizures [6,67]. GABA_A receptors are therefore widely used in the treatment of anxiety
disorders, insomnia, epilepsy, restlessness, and aggressive behaviours [17,68]. Recently, Rudolph and Möhler [69] gave comprehensive overview of the role of GABA_A receptor subtypes on cognitive and emotional behaviour, particularly to cognitive dysfunction and Down syndrome, anxiety disorders, depression, schizophrenia, and autism. They highlighted that a partial inverse agonist acting at the α5-subunit-containing GABA_A receptors is in a clinical trial in individuals with Down syndrome. Substances that selectively reduce function of α5-containing GABA_A receptors are considered as potential cognition enhancers for Alzheimer's syndrome and other dementia, consistent with genetic studies implicating GABA_A receptors in learning performances [70]. With regard to anxiety disorders, non-sedative anxiolytics based on the modulation of α2- and α3-subunit-containing GABA_A receptors have been established in clinical proof-of-concept trials. Furthermore, Rudolph and Möhler [69] emphasized the GABA hypothesis of depression and new options for antidepressant drug development. They also pointed out that cognitive symptoms in schizophrenia are attributed to a cortical GABAergic deficit, and that dysfunctional GABAergic inhibition contributes to the pathophysiology of autism spectrum disorders.

Furthermore, the properties of GABA_A receptors specific to interneurons may differ significantly from those found on projecting neurons, offering the possibility of developing interneuron-specific drugs that is of great therapeutic interest as more and more neurological and psychiatric disorders are linked to malfunction or deficits of interneurons [3]. Cells of glial origin also express GABA_A receptor subunit isoforms and form functional ion channels that are modulated by classical GABA_A receptor drugs, including diazepam and anesthetics etomidate and propofol [71].

**Effects of GABA and GABA agonists at GABA_A receptor**

Besides GABA, other compounds also act at GABA recognition sites. Muscimol, 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridine-3-ol (THIP, a bicyclic muscimol analog) and isoguvacine are structural analogs of GABA, and have direct GABA mimetic effects, while alkaloid bicuculline competitively antagonises effects of GABA at GABA binding sites. Muscimol has been widely used as a valuable tool to study pharmacology of GABA_A receptors, and was the lead compound in the development of a range of GABAergic agents [72-74]. Behavioural effects of muscimol and THIP are preferentially mediated via high-affinity agonist binding sites at extrasynaptic receptors containing α4 and δ subunits. For
THIP at high concentrations, the superagonistic behaviour on α4β3δ receptors is achieved due to its ability to increase the duration of longer channel openings and their frequency, resulting in longer burst durations, while muscimol-related moderate superagonist behaviour is caused by reduced receptor desensitisation [75]. The ability to specifically increase the efficacy of extrasynaptic receptor activation by selected exogenous agonists might have important therapeutic implications, particularly under circumstances when synaptic inhibition is compromised, enabling manipulation of different components of inhibitory circuits [75-77]. Furthermore, it was found that chronic activation of GABA_A receptors by muscimol may exert neuroprotective effects against Aβ-induced apoptotic neuronal death [78].

Radioligand binding studies indicated that GABA and GABA agonists stimulate binding of benzodiazepine agonists, and inhibit binding of inverse agonists at benzodiazepine binding site. Vice versa, GABA binding is stimulated by benzodiazepines, barbiturates, neuroactive steroids and anesthetics. Picrotoxin and other convulsants allosterically inhibit binding of GABA at GABA_A receptors [79-83]. Studies with [3H]muscimol and [3H]GABA have revealed two kinds of agonist binding sites on the GABA_A receptor that differ in affinity. Biochemical and pharmacological experiments have suggested that high- and low-affinity sites might be interchangeable conformational variants of the same receptor complex. In most studies, functional responses to GABA have been observed only at micromolar concentrations, while nanomolar concentrations are needed to occupy the high-affinity binding sites. Hence, it is possible that the high-affinity sites represent a desensitized form of GABA_A receptors or otherwise non-functional binding sites [76,84].

The binding site for GABA at GABA_A receptor is located at the extracellular interface between the α and the β subunit, and a number of amino acids residues in the binding pocket have been identified that are important for pharmacological activity, subtype selectivity, assembly, trafficking and surface expression [84-87]. At the major, and the best characterized subtype of GABA_A receptor that is assembled from α, β and γ subunits, there are two binding sites for GABA. When two molecules of GABA bind to receptor complex, the receptor channel opens, and chloride ions rush into the postsynaptic neuron. However, as one pair of these otherwise identical subunits is flanked by γ and β (site 1) and the other by α and γ (site 2), different environments affect the binding sites. In α1β2γ2 receptors, site 2 has an approximately threefold higher affinity for GABA than site 1, whereas muscimol and bicuculline show some preference for site 1. Opening of the chloride channel also occurs for receptors occupied with a single agonist molecule but is promoted approximately 60-fold in
those occupied by two agonists [88]. After occupancy of GABA binding sites, the large portion of the receptor undergo conformational changes that trap agonist inside the binding pocket, a deep narrowing cleft that probably may constrict during channel gating [64].

The potency of GABA in opening chloride channels is determined by subunit composition. For example, in one study aimed to characterize the potency of GABA at human recombinant δ-containing extrasynaptic GABA_A receptors expressed in Xenopus oocytes, it is found that α4/δ-containing receptors display high sensitivity to GABA, with mid-nanomolar concentrations activating α4β1δ and α4β3δ receptors. In the majority of oocytes expressing α4β3δ subtypes, GABA produced a biphasic concentration-response curve, and activated the receptor with low and high concentrations, while at α4β2δ receptors, GABA exerted low micromolar activity. An analysis of 10 point mutations in the large N-terminal extracellular domains in α4β3δ receptors shows that GABA interacts with amino acids different to those reported for α1β2γ2 GABA_A receptors suggesting that GABA may have different binding modes for extrasynaptic δ-containing GABA_A receptors compared to their synaptic counterparts [89].

Effects of drugs acting at benzodiazepine binding sites at GABA_A receptor

Due to their pharmacological and clinical relevance, benzodiazepines have attracted much attention as allosteric modulators of GABA_A receptors. Various ligands acting at benzodiazepine binding site can enhance (positive modulators or agonists) or reduce (negative modulators or inverse agonists) the actions of GABA on GABAergic transmission with a different range of efficacy (from full to partial). Antagonists are devoid of intrinsic activity, but can inhibit effects of both positive and negative modulators [6].

Benzodiazepines and other substances acting at the benzodiazepine binding site are the most commonly prescribed drugs in therapeutic use due to their desirable anxiolytic, myorelaxant, sedative/hypnotic and anticonvulsant effects. Classical benzodiazepines, such as diazepam and flunitrazepam, do not open channel directly, but allosterically potentiate activation of GABA_A receptor containing either α1, -2, -3 or -5 subunits. This means that after their binding, conformational changes at the receptor confer to increased affinity for channel gating by GABA at both agonist sites. Accordingly, in electrophysiological recordings classical benzodiazepines do not affect the maximal current amplitude, but GABA
concentrations required for channel opening are shifted to lower values. In contrast to them, negative allosteric modulators shift GABA concentration curve to the right [5].

The subunit composition of the GABA$_A$ receptor complex largely determines pharmacological properties at benzodiazepine binding site. In fact, pharmacological profile is predominantly determined by $\alpha$ and $\gamma$ subunits isoforms. It has been shown that different receptor conformations, in particular $\gamma\beta\alpha1\beta\alpha6$, $\gamma\beta\alpha6\beta\alpha1$, $\gamma\beta\alpha1\beta\alpha1$ and $\gamma\beta\alpha6\beta\alpha6$, have their own pharmacological signature and that diazepam efficacy is determined exclusively by the $\alpha$- subunit neighbouring the $\gamma2$-subunit [35]. The most applied concept is that the $\alpha1\beta\gamma2$-receptors, called type I BZ receptors, preferentially bind full benzodiazepine agonists with high affinity, while $\alpha2/3/5\beta\gamma2$-receptors, classified as type II BZ receptors, have approximately 10-fold lower affinity for benzodiazepine agonists [24,81]. Sedative, anterograde amnesic and partly anticonvulsant properties are attributed to $\alpha1$-containing receptors, while the presence of $\alpha2$ and/or $\alpha3$ subunits determines anxiolytic and myorelaxant activity of benzodiazepines [90-92]. This means that compound that selectively modulates receptors with $\alpha2$ and/or $\alpha3$ subunits, but not $\alpha1$-containing receptors, would be a non-sedating anxiolytic, suitable for the treatment of generalised anxiety disorder [93]. Receptors containing $\alpha5$ subunits confer to learning and temporal and spatial memory. The $\alpha5$ subunit of the GABA$_A$ receptor is localized mainly to the hippocampus of the mammalian brain. It is shown that the largely extrasynaptic $\alpha5$ GABA$_A$ receptors in hippocampal pyramidal cells are implicated as control elements of the temporal association of threat cues in trace fear conditioning [94]. Furthermore, the $\alpha5$ -/- mice showed a significantly improved performance in a water maze model of spatial learning, indicating that $\alpha5$-containing receptors play a key role in cognitive processes [95]. However, as pointed by Sigel and Steinmann [5], behaviour is a complex phenomenon, and caution is needed in an attempt to define the role of individual GABA$_A$ receptor subunits, as most probably several types of GABA$_A$ receptors determines even the simple behavioural traits. Receptors containing $\alpha4$ and $\alpha6$ subunits are generally thought as insensitive to classical benzodiazepine agonists [96,97], although they retain a high affinity for the partial inverse benzodiazepine agonist Ro 15-4513 [98]. Unusually, specific high-affinity binding of the benzodiazepine agonist flunitrazepam to $\alpha6\beta\gamma2$-containing GABA$_A$ receptors was shown [99], but flunitrazepam action on $\alpha6\beta\gamma2$-receptors was opposite to its action on $\alpha1\beta\gamma2$-receptors where it exhibits an inverse agonistic effect in a GABA-dependent manner.
Benzodiazepines bind with high-affinity to a binding site located at the α/γ subunit interface in the extracellular domain of GABA_A receptor [65,100]. The binding site for benzodiazepines is formed by one of α subunits (α1, α2, α3 or α5) and a γ subunit (typically the γ2 subunit, which is present in approximately 90% of all GABA_A receptors) [4]. Studies on recombinant GABA_A receptors indicated that co-expression of α, β and γ subunits is required for arrangement of high-affinity binding sites at GABA_A receptor complex [101]. Hanson and Czajkowsky [102] have identified that Gln182-Arg197 region of γ2 subunit is part of the allosteric pathway that allows propagation of structural changes induced by positive benzodiazepine modulators through the protein to the channel domain. Furthermore, several amino acid residues on α and γ subunits have been identified that are crucial for benzodiazepine activity and form benzodiazepine binding pocket [86,103-105]. It is considered that antagonists bind in a pocket partly overlapping with the agonist site, although they can extend further into the solvent accessible cavity [106].

Richter et al. [107] described a binding mode for diazepam and analogs. By using this binding hypothesis and virtual screening approaches, they identified numerous known ligands of benzodiazepine-binding site from different structural classes and predicted potential new chemotypes for this site, thus demonstrating its suitability for drug discovery and structure-based drug design which is of major therapeutic importance. According to the model, although hundreds of ligands from different structural classes can bind to the benzodiazepine-binding sites, only a minority is bound to the same bioactive conformation as diazepam.

Evidence exists that some benzodiazepines can also bind to additional binding sites on GABA_A receptor complex. Walters et al. [108] demonstrated that in the presence of low concentrations of GABA, diazepam produces a biphasic potentiation of α1β2γ2-receptor channels, with distinct components in the nanomolar and micromolar concentration ranges. Mutations at equivalent residues within the second transmembrane domains (TM2) of α, β and γ subunits abolished only the micromolar component, while nanomolar component, which depends on the presence of γ2 subunit, was not affected. Similarly, converse mutation of the corresponding TM2 residue and a TM3 residue within ρ1 subunits confers diazepam sensitivity on homo-oligomeric ρ1-receptor channels that are otherwise insensitive to benzodiazepines. Furthermore, effect of micromolar component on the GABA-induced current was not inhibited by flumazenil, an antagonist of benzodiazepine binding sites. As these transmembrane domains are important for effects of anesthetics, and taking into account anesthetic properties of diazepam, it might be that low potency modulation is elicited via anesthetic binding site [66].
In contrast to diazepam that acts as positive allosteric modulator in both nanomolar and micromolar range, β-carbolines, such as methyl-6,7-dimethoxy-4-ethyl-β-carboline (DMCM), act as negative allosteric modulators at high affinity sites, but potentiate effects of GABA via the low-affinity binding sites [109]. The apparent reversal of inhibition and potentiation by high concentrations of DMCM is insensitive to flumazenil, β2/3 subunit dependent, and probably elicited via the binding site for anticonvulsant loreclezole in the transmembrane domain. Moreover, Baur et al. [110] found evidence for the presence of a third site for benzodiazepines that prevents modulation of GABA_A receptors via the classical benzodiazepine site. The novel site potentially contributes to the high degree of safety to some of these drugs. Their results indicate that this site may be located at the α/β subunit interface pseudo-symmetrically to the site for classical benzodiazepines located at the α/γ interface.

In addition, it is found that the specific effects of imidazobenzodiazepine Ro15-4513 in antagonizing ethanol-stimulated ^36^Cl^-^ uptake and behaviour can be completely blocked by benzodiazepine receptor antagonists. As other benzodiazepine receptor inverse agonists fail to antagonize the actions of ethanol in vitro or in vivo, a novel binding site for Ro15-4513 is also hypothesized [111]. Hanchar et al. [112] demonstrated that extrasynaptic δ subunit-containing receptors bind the imidazo-benzodiazepines flumazenil and Ro15-4513 with high affinity, contrary to the widely held belief that these receptors are benzodiazepine insensitive. Recently, it was identified that in addition to benzodiazepine binding site, Ro15-4513 interacts with high affinity to sites at the α4/6+β3- interface (each subunit has a plus and minus side determined by the absolute arrangement of subunits around central pore) and thus inhibits ethanol effects [98].

As for some potential binding sites no interacting drugs have been identified, Ramerstorfer et al. [113] established a steric hindrance procedure for the identification of drugs acting at the extracellular α1+β3- interface, which is homologous to the benzodiazepine binding site at the α1+γ2- interface, while GABA-binding sites are located at the β+α- interface [66]. Among screened compounds, anxiolytic pyrazoloquinoline 2-p-methoxyphenylpyrazolo[4,3-c]quinolin-3(5H)-one (CGS 9895) was able to enhance GABA-induced currents at α1β3 receptors from rat. Thus, at nanomolar concentrations CGS 9895 behaves as an antagonist of high-affinity, benzodiazepine binding site, but exerts a low-potency positive modulatory action at the extracellular α1+β3- interface of α1β3γ2 and α1β3 receptors. Other experiments indicated that the CGS 9895 effect was dependent on α and β subunit isoforms forming the interface. Since CGS 9895, even at high concentrations, was only able to enhance GABA-induced currents but not directly activate these receptors, it is
likely that drugs acting via the α+β− interface only have GABA-modulatory properties, like the benzodiazepines do. In contrast to benzodiazepines, these drugs will interact with receptors composed of αβ, αβγ, and αβδ subunits. Hence, they would exhibit a much broader anticonvulsive action than benzodiazepines, which might be of clinical importance for the treatment of epilepsy and development of subtype-selective drugs.

Based on results obtained with crystal structure of ELIC, prokaryotic homolog of GABA receptor, it is possible that at least some benzodiazepines, such as flurazepam, may directly interact with the GABA-binding site at β+α- interface at high concentration and displace GABA from its binding site, although these effects are observed only at high concentrations and further experiments are needed to confirm if this site has a functional role in GABA receptors [66,114].

Unfortunately, despite their proven clinical efficacy, positive modulators of benzodiazepine binding sites at GABA receptor possess a relatively narrow window between doses that produce anxiolytic effects and those that cause sedation, and are associated with the development of tolerance and physical and psychological dependence and a potential for abuse [115-117]. Hence, strategy to resolve the disadvantages of classical full agonists was directed toward development of partial agonists with lower intrinsic efficacy. A number of non-selective partial agonists with reduced intrinsic efficacy, including bretazenil, pazinaclone and abecarnil, were described. In general, although they have a large window between anxiolytic and sedative doses and their dependence and abuse liabilities were much lower, they failed to translate into clinical benefit [93]. More recently, the imidazolone derivatives imepitoin was shown to act as low-affinity partial agonist at the benzodiazepine site of the GABA receptor, and was approved for the treatment of epilepsy in epileptic dogs due to its more favorable pharmacokinetic profile in dogs versus humans, reactivating again the interest for partial benzodiazepine agonists for the treatments of human epilepsy [118].

Regarding development of tolerance and dependence to benzodiazepine-site ligands after prolonged administration, diverse effects at the molecular and cellular level have been described, including receptor desensitization and changes in the number and subunit composition of surface GABA receptors, often leading to decreased ability of benzodiazepines to potentiate effects of GABA [17,119-124].

Effects of convulsants at GABA receptor
Very early picrotoxin was identified as a noncompetitive inhibitor of GABA<sub>A</sub> receptor. Binding studies further revealed that numerous compounds from heterogeneous chemical classes posses affinity for picrotoxin-binding sites, including <i>t</i>-butylbicyclophosphorothionate (TBPS), <i>t</i>-butylbicycloorthobenzoate (TBOB), pentylenetetrazole, and some insecticides (such as dieldrin and lindane). They inhibit GABA-induced influx of chloride ions acting as allosteric antagonists at GABA<sub>A</sub> receptor complex [125-128].

TBPS partially and allosterically inhibits binding of GABA and positive modulators of GABA<sub>A</sub> receptors, but facilitates binding of negative modulators. Similarly, modulators of GABA<sub>A</sub> receptor function affect <sup>[35S]</sup>TBPS binding. GABA exhibits biphasic effect on the allosteric modulation of <sup>[35S]</sup>TBPS binding. Thus, <sup>[35S]</sup>TBPS binding was allosterically inhibited by high concentrations of GABA, GABA agonists and other positive modulators (various benzodiazepine receptor agonists, anesthetics and pentobarbital) and promoted in the presence of negative modulators such as ethyl-β-carboline-3-carboxylate (β-CCE). On the contrary, GABA applied at low concentrations enhanced <sup>[35S]</sup>TBPS binding [6,36,129-132]. In addition, the effects of the anesthetic steroid and pentobarbital on <sup>[35S]</sup>TBPS binding were markedly altered by GABA, which at 2 μM increased their maximal effects and half-maximal concentrations. On the contrary, GABA did not affect changes in <sup>[35S]</sup>TBPS binding produced by various benzodiazepine receptor agonists, indicating more pronounced functional coupling of the GABA sites with those for the steroid and the barbiturate, as compared with the benzodiazepine site. Hence, it appears that the degree of <sup>[35S]</sup>TBPS binding in the presence of GABA reflects the functional state of GABA<sub>A</sub> receptors and may be useful for characterization of allosteric interactions between various sites on the receptor [6,133].

Inhibitory effects of GABA on <sup>[35S]</sup>TBPS binding are determined by α subunit isoform. It has been shown that expression of recombinant α6β2γ2 GABA<sub>A</sub> receptors produces <sup>[35S]</sup>TBPS binding sites that are about 10-fold more sensitive to inhibition by GABA than were those inherent to α1β2γ2 GABA<sub>A</sub> receptors. Similarly, the neurosteroid 5α-pregnan-3α-ol-20-one (allopregnanolone) affected the binding in both α1β2γ2 and α6β2γ2 receptors, but inhibition was greater in α6-containing than in α1-containing receptors, also suggesting differential coupling of both GABA and neurosteroid sites with the convulsant site [134].

At first, it was suggested that picrotoxin's site of action is probably within the channel pore as mutations in TM2 domains (that form chloride channels) produced currents that were insensitive to picrotoxin [135]. By convention, Cys-loop receptor TM2 residues are numbered
from intracellular 0 to extracellular 20 positions. Although the exact location of picrotoxin binding to ionophore is still unknown, its sensitivity to mutations in residues 2/3 and 6 of TM2 indicates that the site contains residues 2–6. In particular, it is suggested that picrotoxin enters deep inside the ionophore pore, binds with its hydrophobic moiety to residue 2 of TM2 (close to the pore) and forms hydrogen bonds with residue 6 in the middle of TM2. Residue 15 is probably important for interplay between GABA and picrotoxin binding sites. Other studies implicated residues 9 and 15 in the regulation of channel properties, including desensitization, stabilization of open states and gating. This indirectly supports the possibility of a second “modulatory” (allosteric) binding site of picrotoxin including residues 15–19 [128].

Dillon et al. [136] provided evidence that picrotoxin and TBPS interact with GABA-bound and -resting receptors, but their affinity for GABA-bound receptors is about 10 times greater, largely due to a markedly increased association rate to the multi-ligated receptors. Accordingly, TBPS and picrotoxin are considered as open channel blockers. However, $[^{35}\text{S}]$TBPS binds to recombinant receptors in the absence of GABA [130]. Hence, it does not gain access to its binding site via the open pore but through alternative routes regulated from the agonist binding site [137]. It is shown that picrotoxin displaceable $[^{35}\text{S}]$TBPS binding to $\alpha1\beta2\gamma2$ GABA$_A$ receptors might occur in the absence of GABA, suggesting that access to the binding site is independent of activation. In fact, bicuculline-sensitive spontaneous gating contributes to $[^{35}\text{S}]$TBPS binding in the absence of GABA by providing access to the channel. Accordingly, a decrease in a spontaneous gating reduces accessibility of TBPS to its binding site [132]. GABA application during picrotoxin or TBPS administration enhanced $\alpha1\beta2\gamma2$ receptor blockade, and this GABA-dependent component of TBPS blockade accounts for the stimulation of $[^{35}\text{S}]$TBPS binding to $\alpha1\beta2\gamma2$ receptors seen with low concentrations of GABA, while application of GABA at concentrations that cause significant steady-state desensitization reduces $[^{35}\text{S}]$TBPS binding [132].

**Effects of anesthetics at GABA$_A$ receptor**

General anesthetics are used to induce a reversible loss of consciousness in surgical patients and to relieve pain. GABA$_A$ receptors are the major site of action of clinically used intravenous anesthetics such as etomidate, propofol, barbiturates and neuroactive steroids. On the other hand, clinically used volatile anesthetics such as isoflurane and enflurane, and long-
chain alcohols, presumably act via a multitude of targets, GABA_A receptors being just one of them [4,63,138,139].

In general, GABA_A receptors are positively modulated by clinical doses of anesthetics, while at higher concentrations anesthetics can directly activate GABA_A receptors [63,140]. Accordingly, many studies were aimed to identify binding site(s) for anesthetics on GABA_A receptors, which are generally considered as the main target for propofol and etomidade action as already mentioned. Belelli and co-authors [141] have shown that the ability of etomidade to modulate and activate GABA_A receptors in vitro is uniquely dependent upon the β subunit subtype present within the receptor, i.e. receptors containing β2- or β3-, but not β1 subunit, are highly sensitive to etomidade. They identified a region distal to the extracellular N-terminal domain as a determinant of the selectivity of etomidade: mutation of Asn present within the channel domain of the β3 subunit to Ser (the homologous residue in β1) strongly suppressed the GABA-modulatory and GABA-mimetic effects of etomidade. Later, it was confirmed that the same point mutation (Asn265Met) completely abolished the suppression of noxious-evoked movements in response to propofol and etomidade in a knock-in mouse, while only slightly decreased in response to volatile anesthetics enflurane and halothane. Mice also displayed a profound reduction in the loss of righting reflex duration (a surrogate for loss of consciousness) in response to intravenous but not volatile anesthetics. Accordingly, electrophysiological recordings revealed that anesthetic agents were significantly less effective in enhancing GABA-induced currents in cortical brain slices derived from mutant mice, thus identifying the key molecular determinant of behavioural responses evoked by intravenous anesthetics [142].

Studies on recombinant receptors have revealed that isoforms of α, γ and δ subunits affect propofol modulation of the GABA_A receptor, although it seems likely that the propofol binding site involves the β subunit (see below). In order to investigate the role of α subunit in the modulatory effects of propofol, Krasowski et al. [143] used whole-cell patch clamp recordings from cells expressing only α1β3γ2 or α6β3γ2 subtypes, and found that at clinically relevant concentrations, propofol potentiated submaximal GABA currents in α1β3γ2 receptors to a far greater degree than in α6β3γ2 receptors. Isoform of α subunit influenced the efficacy of propofol for modulation, but not its potency. On the contrary, direct gating of the chloride channel was significantly larger in the α6β3γ2 receptors. A same trend for potentiation and direct gating was observed for other anesthetics. Similarly, pharmacological properties of human recombinant GABA_A receptors were studied in Xenopus oocytes expressing α1β2,
α1β2γ2L, or α2β2γ2L receptor isoforms. In all receptor isoforms tested, propofol potentiated the GABA-activated currents in a concentration-dependent manner, and was able to directly activate all three receptor isoforms. Addition of the γ2L subunit to the α1β2 isoform reduced receptor sensitivity to direct activation, while replacement of the α1-subunit with the α2-subunit increased receptor sensitivity to propofol's direct effects, indicating that isoforms of α and γ subunits have the ability to influence both the direct and modulatory actions of propofol on GABA<sub>A</sub> receptor function [144]. Jones et al. [140] reported that γ2 subunit is not a prerequisite for activation of GABA<sub>A</sub> receptors by propofol or for its potentiation of GABA-activated currents, but showed that it may contribute to the efficacy of propofol as a GABA<sub>A</sub> receptor activator. Furthermore, the findings that propofol reduced desensitization and prolonged deactivation of receptors containing γ2L subunit and enhanced peak currents or prolonged deactivation of receptors containing δ subunit suggest that propofol-induced enhancement of both phasic and tonic inhibition may contribute to its anesthetic effect in the brain [145]. In accordance with mentioned studies, mutations in α and γ subunits change modulatory effects of propofol at the GABA<sub>A</sub> receptors [146,147], while particular residues in β subunits, distributed throughout the membrane-spanning region of the receptor, as well as in the extracellular domain, determine the propofol’s actions. Thus, a point mutation in the β1 subunit (Met286Trp) abolished potentiation of GABA by propofol, but did not alter direct activation of the receptor by high concentrations of propofol [148]. The same mutation had the same effect in the β2 subunit, probably by altering the dimensions of a binding pocket for propofol and related alkylphenol general anesthetics [149]. One mutation in β2 subunit (Tyr444Trp) was particularly interesting, as it selectively suppressed the ability of propofol to enhance receptor function, while retaining normal sensitivity to etomidate [150].

Although various anesthetics do not bind to the same binding site and exhibit distinct structural requirements for modulatory and direct actions, it seems that main sites of action are located close to each other, within the transmembrane domain [151]. The influence of the large intracellular loop in propofol sensitivity has also been recognized. Moraga-Cid et al. [147] have shown that mutation of a conserved Phe385 residue within the α1 large intracellular loop significantly reduced propofol enhancement, although propofol-hyposensitive mutant receptors retained their sensitivity to other allosteric modulators such as alcohols, etomidate, trichloroethanol, and isoflurane. At the single-channel level, the ability of propofol to increase open probability was significantly reduced, altogether suggesting a new role of the large intracellular loop in allosteric modulation, and providing new insight into the propofol-mediated modulation of GABA<sub>A</sub> receptor complex. By using a novel propofol
analogue photolabeling reagent, Yip et al. [152] have identified a novel binding site for propofol in β3 homopentamers and α1β3 heteropentamers. The binding site is located within the β subunit, at the interface between the transmembrane domains and the extracellular domain, and lies close to known determinants of anesthetic sensitivity in transmembrane segments TM1 and TM2.

The similar approach was used to determine binding site for etomidate. By using radiolabeled, photoreactive etomidate analogs, residues αMet-236 and βMet-286 in the αTM1 and βTM3 transmembrane helices, were identified as a part of single binding pocket in the transmembrane domain at the β+α- interface, close to the extracellular domain, bellow the GABA-binding site. Such localization of the etomidate binding site to an inter-subunit, not an intra-subunit binding pocket was an interesting finding, suggesting that binding sites at subunit interfaces may be a feature not only for GABA and benzodiazepines, but also for etomidate and other anesthetics [153,154]. As photolabeled etomidate analog was also displaced by isoflurane, Li et al. [139] suggested that inhalation anesthetics also interact with this binding site. More precisely, in their study photolabeling was inhibited by low millimolar anesthetic concentrations of propofol, barbiturates, and isoflurane, but not by octanol or ethanol. Inhibition by barbiturates and propofol was only partial, consistent with allosteric interactions, whereas isoflurane inhibition was nearly complete, suggesting competitive interactions.

Effects of barbiturates at GABA<sub>A</sub> receptor

Response of GABA<sub>A</sub> receptors may also be modulated by barbiturates. They have been used in the past due to their anticonvulsant, anxiolytic, sedative, and hypnotic actions, but they exert serious side effects, including profound depression of CNS activity with the induction of pronounced sedation, cognitive slowing or confusion, hyperactivity in children and a lethal risk in a case of overdose [155-157].

As they bind to GABA<sub>A</sub> receptor with low affinity, most of their actions have been analysed indirectly by studying interactions with other binding sites. Barbiturates enhance binding of GABA and benzodiazepines, whereas inhibit binding at convulsant binding site [158]. Depending on the concentration, barbiturates act in three different ways, and their effects are proportional to anesthetic potency. At low µM (sub-anesthetic) concentrations they potentiate GABA-induced effects (modulatory effect) and prolong duration of open conformation of chloride channel, possibly by stabilizing open state(s). In higher µM
(anesthetic) concentrations (app. >50 µM) they directly open chloride channel (agonistic effect), whereas at very high mM concentrations block GABA-induced current (inhibitory effect) [159,160]. More precisely, when co-applied with low concentrations of GABA, barbiturates increased the mean open time. The increase in channel open time results in greater chloride current flux and increased likelihood that channel openings will summate, producing larger inhibitory currents [157]. In the absence of GABA, high concentrations of barbiturates may directly activate GABA<sub>A</sub> receptor chloride currents, although with lower efficacy than GABA [60]. All these effects are probably mediated by separate mechanisms. In support of this conclusion, it was demonstrated that mutation of Thr262 in the transmembrane domain of β1 subunits at α1β1 recombinant GABA<sub>A</sub> receptors abolished modulatory effect, without affecting direct agonistic or inhibitory effects of pentobarbital [161].

Although barbiturate sensitivity does not require a specific subunit composition, their effects are also determined by subunit composition [57,157]. Isoforms of α subunit affect efficacy, but not affinity of barbiturates in potentiation of GABA response. In contrast to benzodiazepines that are ineffective at GABA<sub>A</sub> receptors containing α4 or α6 subunit, barbiturates elicit modulatory effects at receptors containing these isoforms [97,159], and the isoform of α subunit influences the level of potentiation [162]. Related to agonistic activity, the type of α subunit present determines both the degree of affinity and efficacy observed. Receptors containing α6 isoform produce maximum direct response to barbiturate pentobarbitone, larger than that obtainable with maximum GABA, and larger than that obtainable with other α subunits. GABA<sub>A</sub> receptors containing α6 subunits also have higher affinity for direct activation by pentobarbitone. Furthermore, the direct effect of pentobarbitone can be blocked by picrotoxin, but not by competitive antagonists, such as bicuculline [97,159]. Surprisingly, unlike receptors containing α6 isoform, α4β1γ2 receptors did not elicit any direct activation of GABA<sub>A</sub> receptors by pentobarbital [97].

Some amino acid residues have been identified with major impact on barbiturate activity. An invariant proline residue in transmembrane domain TM1 is included in a consensus motif of all GABA<sub>A</sub> receptor subunits. Proline in TM1 segment of β1 subunit affects the linkage between GABA binding and channel gating and is critical for barbiturate enhancement at recombinant GABA<sub>A</sub> receptors, without affecting enhancement by diazepam or neurosteroid alfaxalone [163]. Gly219 near the TM1 at β2 subunit is also identified as important for allosteric (modulatory) effects of pentobarbital since pentobarbital-induced enhancement of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam binding in receptors containing the β2(Gly219Phe) point mutation displayed a significantly reduced efficacy in modulation.
Furthermore, functional analysis of pentobarbital-enhanced GABA currents recorded with whole-cell patch clamp demonstrated that this mutation eliminates the potentiating effect of the anesthetic. Interestingly, the α1β2(Gly219Phe)γ2 receptors also were more sensitive to direct channel activation by pentobarbital, suggesting that Gly219 may be important for conformational or allosteric interactions of channel gating by GABA and barbiturates [164].

Chiara et al. [165] used a photoreactive barbiturate analog to determine residues important for barbiturate binding site. It photolabeled sites at the α+β- and γ+β- subunit interfaces in the transmembrane domain, near the synaptic side. These binding sites are distinct but homologous to the etomidate sites at the two β+α- subunit interfaces, as all are located at the same depth in the transmembrane domain. They demonstrated that photoreactive barbiturate and etomidate derivatives are highly selective for their own sites, indicating that there are two structurally related, but pharmacologically distinct, classes of inter-subunit general anesthetic-binding sites in the transmembrane domain of human α1β3γ2 GABA_A receptors. They hypothesized that binding at any of these homologous inter-subunit sites is sufficient for anesthetic action. Namely, a wide range of general anesthetic structures target these four sites but with variable selectivity, which offers an explanation of the puzzling lack of well defined structure-activity relationships among general anesthetics. More importantly, these findings indicate that it may be possible to synthesize general anesthetics with specificity for sites between specific subunits in the transmembrane domain of pentameric GABA_A receptors, with a hope to target specific nerve pathways and behaviours in a subunit-dependent manner in the future [165].

**Effects of neurosteroids at GABA_A receptors**

Inhibitory neurotransmission mediated by GABA_A receptors can be modulated by endogenous neurosteroids, mostly metabolites of progesterone and deoxycorticosterone, including allopregnanolone and tetrahydrodeoxycorticosterone (THDOC). In general, they potently enhance function of synaptic and extrasynaptic GABA_A receptors by an allosteric mechanism [31,40,166,167]. Neurosteroids possess distinct, characteristic effects on the membrane potential and current conductance mainly via potentiation of GABA_A receptors at low doses, and direct activation of receptor chloride channel at higher concentrations. As a result, upon administration neurosteroids exert anxiolytic, analgesic, anticonvulsive, sedative and hypnotic effects, while applied at higher doses may induce a state of general anesthesia.
Although the most important effects of neurosteroids are mediated via GABA_A receptors, they also exert various effects on an array of ligand-gated ion channels and distinct G-protein-coupled receptors via nongenomic mechanisms, including the N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate, glycine, serotonin, sigma type-1, and nicotinic acetylcholine receptors [156,172]. Besides modulation of diverse receptors, the other functions attributed to specific neurosteroids include neuroprotection, for example against glutamate-induced excitotoxicity, and induction of neurite outgrowth, dendritic spines and synaptogenesis, and contribution to the behavioural effects of psychoactive drugs [49,51,173-175].

As emphasized before, neurosteroids exhibit two modes of action at GABA_A receptors. They allosterically modulate channel opening, and at high concentrations act as GABA-mimetics and directly open the chloride channel [31,53,176]. Two discrete binding sites in the receptor’s transmembrane domains have been identified that mediate the potentiating and direct activation effects of neurosteroids. Neurosteroids potentiate GABA responses from a cavity formed by the α-subunit transmembrane domains, while direct receptor activation is achieved by interfacial residues between α and β subunits and is additionally enhanced by steroid binding to the potentiation site, indicating that activation of GABA_A receptors by neurosteroids relies on the occupancy of both sites [177]. Further work provided a more detailed kinetic and pharmacological characterization of the effects of mutations on channel activation and modulation by neurosteroids, leading to a model where residues in the TM1 membrane-spanning domain shape the binding surface to accommodate a variety of structurally distinct neurosteroids [178]. The potentiating effect of neurosteroids can be mediated by steroid interactions with its site within the same β-α pair that mediates receptor activation as well as the opposite β-α pair [179]. Chisary et al. [180] have shown that neurosteroids require a membranous route of access to transmembrane-domain binding sites that might have implications for the design of novel neuroactive steroids because their lipid solubility and related accessibility are probably the key determinants of receptor modulation [180]. This also suggests that by virtue of their high lipid solubility, µM concentrations of neurosteroids may be achieved locally [31].

Neuroactive steroids may act both as positive and negative modulators of GABA_A receptor function. Applied at concentrations below 300 nM, they are positive allosteric neuromodulators as they facilitate conformational transition of the GABA-gated chloride channel to an open state, increase mean channel open time and consequently increase GABA-elicited chloride currents [49,131,181-183]. Furthermore, they modulate binding of GABA
agonists, increase binding of positive modulators at benzodiazepine binding sites, and enhance TBPS binding in the absence of GABA [184-187]. On the contrary, sulphated endogenous steroids like pregnenolone sulphate and dehydroepiandrosterone sulphate (DHEAS) act as negative modulators at steroid binding site when applied at µM concentrations [59]. For various neuroactive steroids sulfation at C-3 reverses the direction of modulation from positive to negative, suggesting that sulfation could be an important control point for the activity of endogenous neurosteroids. As interactions of positive and negative steroid modulators are not competitive, they probably act via distinct binding sites at GABA_A receptor. This implies that negative and positive steroid modulators can act independently or coordinately to regulate GABA-mediated inhibition in the CNS [188]. Antagonistic neurosteroids may shorten the channel open time and enhance GABA-induced desensitization [6].

Similarly to other binding sites, subunit assembly determines sensitivity and pharmacological effects of neuroactive steroids. Studies on recombinant GABA_A receptors have revealed that presence of δ subunit confers increases sensitivity to neurosteroid modulation by affecting intrinsic gating and desensitization kinetics [181,189]. Thus, at concentrations known to occur in vivo, neuroactive steroids specifically enhance a tonic inhibitory conductance in central neurons that is mediated by extrasynaptic δ subunit-containing GABA_A receptors [190]. In addition, expression of extrasynaptic GABA_A receptors is dynamically regulated by neuroactive steroids. For example, it is suggested that neuroactive steroids mediate a stress-induced enhancement in the expression of α4βδ receptors, probably contributing to observed stress-related memory impairment [41,44,191]. Potency and efficacy of neuroactive steroids is also determined by other subunits. Although these differences are not very pronounced, even small differences in receptor sensitivity might be physiologically relevant. Namely, allopregnanolone concentrations in plasma are 3-10 nM in physiological conditions, and increase only to 30-60 nM during stress [168].

Interestingly, it is confirmed that endogenous neurosteroids profoundly affect neuronal activity at particular receptor isoform. For example, it is shown that α1β2δ subtype of GABA_A receptors presumably expressed in the forebrain, exhibits an extremely small GABA-mediated current in the absence of the modulator, but in the presence of THDOC exerts a profound inhibitory influence on neuronal activity by increasing maximum current amplitude and decreasing half-maximal concentration (EC_{50}) of GABA [192]. Hence, depending on the subunit composition, GABA-modulatory effects of physiological levels of the neurosteroid are not uniformly experienced throughout the CNS, or even within the same brain region. In
In addition, these modulatory effects are determined by the phosphorylation status of the GABA_A receptor, or associated proteins, and by local steroid metabolism [31,49,54,193].

As endogenous ligands, neuroactive steroids may modulate diverse physiological and pathophysiological conditions, regulating GABA_A receptor-associated functions and behaviour. Altered neurosteroid levels are associated with psychiatric and neurological disorders, including premenstrual dysphoric disorder, premenstrual syndrome, menstrual migraine, postpartum depression, pain disorder, catamenial epilepsy, major depression, schizophrenia, alcohol dependence and anxiety [51,177,194]. The pathogenesis of these diseases remains unclear, partly due to the lack of useful animal models to study such complex disorders [46]. As mentioned before, pregnancy is associated with a pronounced enhancement in progesterone-derived neurosteroid levels, with sharp decline after delivery. Because neuroactive steroids exert anxiolytic effects, neurosteroid withdrawal could play role in the pathophysiology of postpartum depression [195]. Synthetic neurosteroids that exerts better bioavailability and efficacy, as well as drugs that enhance neurosteroid synthesis and lack unwarranted side effects of benzodiazepines, are therefore considered as potential therapeutics in the treatment of anxiety, epilepsy and other brain disorders [156,195-197].

**Effects of ethanol at GABA_A receptors**

Ethanol is often classified as modulator of GABA_A receptor activity. It is without doubt that very high concentrations of ethanol modulate activity of diverse membrane proteins, including GABA_A receptors [198]. However, it is not quite clear whether social ethanol intake (<20 mM) really exerts effects on human behaviour via GABA_A receptors [5]. Wallner and co-authors [199] have demonstrated that GABA_A receptors might be enhanced by ethanol concentrations that are reachable by moderate, social consumption. They found that GABA_A receptors responsive to these low concentrations require presence of δ-subunit, which is thought to be associated exclusively with α4- and α6-subunits, and the β3-subunit. Their finding also suggests that extrasynaptic GABA_A receptors are primary targets for ethanol. Later, Borghese and co-workers [198] failed to replicate the sensitivity of α4β3δ GABA_A receptors to low concentrations of ethanol at rat and human recombinant GABA_A receptors expressed in Xenopus oocytes. In addition, in their study ethanol at 30 mM concentration did not affect tonic GABA-mediated currents in dentate gyrus reported to be mediated by α4β3δ subtype of GABA_A receptors. Similarly, in another study, α6β3δ GABA_A
receptors were expressed in Xenopus oocytes and were not modulated by physiological concentrations (up to 30 mM) of ethanol [200].

On the other hand, prolonged ethanol consumption that leads to the development of alcohol dependence induces changes of GABA$_A$ receptor subunits at transcriptional and translational levels in brain area-specific manner, including reduction in $\delta$ subunit expression in the orbitofrontal cortex, cerebellum, hippocampus and amygdala. Changes in receptor composition might have important consequences for GABAergic neurotransmission, plasticity and behaviour including impairment in memory, anxiety, and executive and motor functions [201-203]. Furthermore, it was found that ethanol at concentrations of 20 to 100 mM stimulates GABA-mediated uptake of chloride ions in isolated brain vesicles, and this effect can be blocked by the imidazobenzodiazepine Ro15-4513. Pre-treatment of rats with Ro15-4513 blocks the anticonflict activity of lower doses of ethanol, and behavioural intoxication observed with higher doses of ethanol. These effects of Ro15-4513 in antagonizing ethanol-stimulated $^{36}$Cl$^-$ uptake and behaviour can be completely blocked by benzodiazepine binding site antagonists, indicating that neuropharmacological actions of ethanol (20-100 mM) may be mediated via central GABA receptors [111]. Similarly, it was shown that $[^3H]$Ro15-4513 binding is inhibited only by those benzodiazepine-site ligands that reverse the behavioural alcohol antagonism of Ro15-4513 such as flumazenil and $\beta$-carboline-3-carboxylate ethyl ester ($\beta$-CCE), but not by any classical benzodiazepine agonists. Additional experiments indicated that ethanol and Ro15-4513 occupy mutually exclusive binding sites. Since only Ro15-4513, but not flumazenil, can inhibit ethanol effects, and taking into account that Ro15-4513 differs from flumazenil by only a single group at the C7 position of the benzodiazepine ring, it is assumed that this group in Ro15-4513 might be the area that overlaps with the alcohol-binding site. Hence, it turns out that many of the behavioural effects of ethanol at relevant physiological concentrations are mediated via ethanol/Ro15-4513-sensitive GABA$_A$ receptors [112].

Wallner and co-workers [98] showed that differences in alcohol sensitivity toward $\beta$ subunits are determined by the extracellular N-terminal part of the protein. By using point mutations, they discovered that the $\beta3$ alcohol selectivity is determined by a single amino acid residue in the N-terminus that differs between $\beta$ subunits ($\beta3$Tyr66, $\beta2$Ala66, $\beta1$Ser66). The $\beta3$Tyr66 residue is located in a region which in $\gamma$ subunits contributes to the imidazobenzodiazepine binding site at the classical $\alpha$+$\gamma2$- subunit interface. Furthermore, they proposed a model in which $\beta3$ and $\delta$ containing GABA$_A$ receptors form a unique ethanol site at the $\alpha4/6$+$\beta3$- subunit interface. As this site is homologous to the classical benzodiazepine
binding site, it also has high affinity for a few selected benzodiazepine site ligands including alcohol antagonistic Ro15-4513 that bears large moiety at the C7 position of the benzodiazepine ring. They also suggested that large moieties at the C7-benzodiazepine ring compete with alcohol for its binding pocket at a α4/6+β3- ethanol/Ro15-4513 site, thus finally providing explanation for the competitive relationship between ethanol and imidazobenzodiazepine antagonists.

**Interactions of flavonoids with GABA\(_A\) receptor**

Flavonoids represent a heterogeneous class of plant secondary metabolites. They exert a wide-range of health-promoting effects including antioxidative, anti-inflammatory, cardioprotective, anticarcinogenic, antidiabetic and neuroprotective activities [204-206]. Like many neuroactive drugs, they achieve their effects, at least in part, by modulating GABA\(_A\) receptors [207,208]. Radioligand binding studies indicated that flavonoids exert a selective affinity for the benzodiazepine binding site at GABA\(_A\) receptor complex, mostly acting as partial agonists [209]. Although it is initially thought that they act on classical, “high-affinity” benzodiazepine binding sites, many flavonoid actions are not inhibited by classical benzodiazepine antagonist flumazenil. For example, Hall et al. [210] have shown that 6-methoxyflavanone and 6-methoxyflavone act as flumazenil-insensitive positive allosteric modulators of GABA responses at human recombinant \(\alpha_1\beta_2\gamma_2\) and \(\alpha_2\beta_2\gamma_2\) GABA\(_A\) receptors. Functional electrophysiological studies suggest that flavonoids act on GABA\(_A\) receptors via two separate mechanisms: by acting on flumazenil-sensitive high-affinity site and an alternative site that may be the flumazenil-insensitive low-affinity benzodiazepine site [208]. However, as in aforementioned study 6-methoxyflavanone inhibited \([^3H]-\text{flunitrazepam}\) binding whilst remaining unaffected by flumazenil, this might suggest a novel allosteric binding site that is independent of both the high-affinity and low affinity benzodiazepine binding sites [210].

More importantly, some flavonoids may directly open certain subtypes of GABA\(_A\) receptors in the absence of GABA [208]. Behavioural studies demonstrated anxiolytic effects of flavonoids in animal models. Some of the tested flavonoids (e.g. chrysin) have anxiolytic effects similar to diazepam, but not associated with myorelaxant, sedative or amnesic actions, suggesting that they could lead to improved therapeutic drugs in the treatment of anxiety [211,212]. However, in addition to positive modulation, flavonoids may also achieve negative
effects on GABA_\text{A} receptors [213,214]. Some flavonoids are positive neuromodulators when applied in the presence of low concentrations of GABA, but in the presence of high concentrations of GABA act as negative modulators [215]. A range of natural flavonoids act as negative modulators of receptors containing \( \rho \)-subunits.

Recently, it was found that quercetin and its glycosides inhibit GABA-induced inward current at recombinant GABA_\text{C} receptors. These inhibitory effects of quercetin and quercetin-glycosides on GABA-induced inward current were noncompetitive and membrane voltage-insensitive, indicating that quercetin and its glycosides regulate GABA_\text{C} receptor channel activity through interaction with a different site from that of GABA [216]. This study also provides evidence that the number of carbohydrate attached to quercetin might play an important role in the regulation of GABA_\text{C} receptor channel activity. On the contrary, 6-methoxyflavanone and 6-methoxyflavone were inactive as modulators at human recombinant receptors containing \( \rho \text{l} \) subunits [210].

Interestingly, menthol, a naturally occurring compound in the essential oil of mint leaves, also act as a positive allosteric modulator of recombinant GABA_\text{A} receptors. In particular, menthol (150-750 \( \mu \text{M} \)) produced a concentration-dependent prolongation of spontaneous GABA_\text{A} receptor-mediated inhibitory postsynaptic currents in the periaqueductal grey (PAG) neurons, although menthol actions were unaffected by the benzodiazepine antagonist flumazenil. Menthol also enhanced a tonic current, which was sensitive to the GABA_\text{A} receptor antagonists picrotoxin and bicuculline, but unaffected by gabazine and GABA_\text{C} receptor antagonist (1,2,5,6-tetrahydropyridine-4-yl)-methyl-phosphonic acid (TPMPA). In addition, menthol potentiated currents induced by the extrasynaptic GABA_\text{A} receptor agonist THIP, altogether indicating that menthol positively modulates both synaptic and extrasynaptic populations of GABA_\text{A} receptors in native PAG neurons [217].

**GABA_\text{C} receptors**

GABA_\text{C} receptors are considered as a specific subtype of GABA_\text{A} receptors due to their structural homology, although they differ from GABA_\text{A} receptors by biochemical, pharmacological and physiological properties [218-220]. They are predominantly expressed in the retina where play a unique functional role in retinal signal processing, but are also expressed throughout brain and in periphery [221,222]. GABA_\text{C} receptors are involved in numerous processes across the CNS, including vision, olfactory senses, sleep, memory and cognitive functions, hormone secretion and pain perception, with promising potential for the
treatment of myopia, sleep disorders, memory and learning enhancement, and fear and anxiety-related disorders [222,223]. Although understanding of the role of GABAC receptors and processes triggered by ligand–receptor interactions in neurons are still limited because of the lack of adequate pharmacological tools, some progress has been made following synthesis and pharmacological evaluation of the selective fluorescent and biotinylated probes for ρ1 GABAC receptors that hopefully will bring new knowledge regarding the binding site and GABAC receptors flexibility, and be useful tools for localizing, visualizing, and studying the physiopathological processes of GABAC receptors [224].

In mammals, there are three isoforms of ρ subunits (ρ1-ρ3) that form homooligomeric (formed by ρ1, ρ2 or ρ3 isoform) or pseudoheteromeric (made up of a combination of ρ1 and ρ2 isoforms or ρ2 and ρ3 isoforms) GABAC receptor pentamers [220,224]. In humans, only two isoforms are expressed: ρ1 and ρ2.

GABAC receptors are sensitive neither to bicuculline (characteristic ligand of GABA A receptors) nor to baclofen (characteristic ligand of GABA B receptors) [222,225]. They are more sensitive to GABA than GABA A receptors, and at least three GABA molecules are required to activate the GABAC receptor [222]. When activated, they have a smaller chloride conductance, longer channel opening time and desensitize less readily in the presence of GABA then GABA A receptors [218]. Benzodiazepines and barbiturates do not modulate GABAC receptors [218]. Neuroactive steroids may modulate GABA-induced current at GABAC receptors, in particular at ρ1 receptor channels, in both positive and negative manner, although the modulation occurs with relatively high concentrations of neuroactive steroids and is more prominent in the presence of low concentrations of GABA [226]. Characteristic agonists of GABAC receptors are cis enantiomer of 4-aminocrotonic acid (CACA) and (+)cis-2-aminomethylcyclopropane carboxylic acid ((+)-CAMP) [218]. Furthermore, GABAC receptors are much less sensitive to GABA A receptor antagonist gabazine, but can be selectively antagonized by (1,2,5,6-tetrahydropyridine-4-yl)-methyl-phosphinic acid (TPMPA), 3-aminopropyl(methyl) phosphinic acid (3-APMPA), and 3-aminopropylphosphonic acid (3-APA), indicating that agonist/antagonist binding pockets of GABA A and GABAC receptors are not the same [220,227,228]. Compound imidazole-4-acetic acid exhibits a ρ subunit-dependent pharmacological profile: at ρ1 and ρ3 receptors acts as a potent antagonist, while at ρ2 receptors as a potent partial agonist [222].

N-terminal half of the ρ subunits has been shown to mediate formation of homo- and heterooligomeric GABAC receptors, and specific sequence within the N-terminus of the ρ1 subunit involved in the assembly process has been determined [229]. Furthermore, several
key structural elements that determine specific pharmacological response of GABA<sub>C</sub> receptors have been found [228]. Namely, mutational studies, including those directed toward N-terminal domain and transmembrane domain TM4, have revealed residues that change sensitivity to agonists or make GABA<sub>C</sub> complex inactive, and contribute to the binding pocket determining properties of GABA binding [221,230,231]. Thus, Tyr102 at ρ1 subunit was identified as part of GABA binding domain, and probably the important residue for coupling agonist binding to channel opening [232]. Barbiturate sensitivity was imparted by mutation of Trp328 at ρ1 subunit, located within the transmembrane domain TM3. It also seems that this residue plays an important role in agonist-dependent activation, suggesting a functional interconnection between the GABA and pentobarbital activation domains [233]. GABA<sub>C</sub> receptors that contain ρ2 subunit exhibit decreased sensitivity to picrotoxin [234]. On ρ1 receptor it is shown that the mechanism of picrotoxin effects is compatible with an allosteric inhibition and receptor activation was a prerequisite for antagonism [235]. Difference in picrotoxin sensitivity of ρ1 and ρ2 homo-oligomers is determined by a single residue. Interestingly, it was found that this amino acid in the putative channel domain (TM2) of GABA ρ1 receptors influences picrotoxin sensitivity, and also meditates agonist binding by an allosteric mechanism [236].

**GABA<sub>B</sub> receptors**

Metabotropic GABA<sub>B</sub> receptors are G-protein coupled receptors that mediate slow and prolonged inhibitory neurotransmission in the brain [237]. They are widely expressed and distributed in the CNS, although GABA<sub>A</sub> sites generally outnumber GABA<sub>B</sub> sites [238]. GABA<sub>B</sub> receptors differ from GABA<sub>A</sub> receptors by their structural and functional properties, but also exhibit numerous pharmacological effects, including central muscle relaxation, epileptogenesis, suppression of drug craving, antinociception, cognitive impairment and inhibition of hormone release [12,239].

GABA<sub>B</sub> receptors can be located presynaptically and postsynaptically. Stimulation of presynaptic GABA<sub>B</sub> receptors decreases conductance of calcium ions via voltage-gated calcium channels. Consequently, activation of presynaptic GABA<sub>B</sub> autoreceptors induces inhibition of GABA release, while presynaptic GABA<sub>B</sub> heteroreceptors suppress release of other neurotransmitters and bioactive peptides. Depending on whether synaptic terminal releases an inhibitory or excitatory neurotransmitter, presynaptic GABA<sub>B</sub> receptors will facilitate or suppress neuronal excitability thus playing important role in tuning various
synapses [12,240,241]. On the other hand, postsynaptic GABA\textsubscript{B} receptors are coupled via G-proteins to inwardly rectifying potassium channels and underlie slow inhibitory postsynaptic currents. Postsynaptic GABA\textsubscript{B} receptors stimulate efflux of potassium ions that hyperpolarizes neuronal membrane and shunts excitatory currents. As presynaptic and postsynaptic GABA\textsubscript{B} receptors are dominantly located at extrasynaptic sites, their activation requires patterns of presynaptic activity that ends in simultaneous GABA spillover from neighbouring synapses and elevations of ambient GABA, as it occurs during epileptic seizures. Furthermore, the association of GABA\textsubscript{B} receptors with glutamatergic synapses suggests their important role in the modulation of glutamatergic neurotransmission [240,242,243].

The GABA\textsubscript{B} receptor was the first heteromeric G-protein coupled receptor identified. It functions as an obligatory heterodimer assembly: both GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits are necessary to form a functional GABA\textsubscript{B} receptor, and neither of these subunits is functional on its own [244] (Kaupmann et al. 1998). Soon after the cloning of both subunits, it was demonstrated that GABA\textsubscript{B2} is required for GABA\textsubscript{B1} to reach the cell surface by masking an endoplasmic reticulum retention signal of GABA\textsubscript{B1} [245]. However, GABA\textsubscript{B2} is not only required for the correct trafficking of GABA\textsubscript{B1}, but also for the proper functioning of the receptor. In particular, GABA\textsubscript{B1} is involved in ligand recognition via its N-terminal extracellular domain and it binds GABA [246]. Although GABA\textsubscript{B2} subunit does not constitute a binding site for any natural GABA\textsubscript{B} ligand [247], it enhances agonist affinity [248], and is required for receptor activation. In addition, GABA\textsubscript{B2} subunit is responsible for G-protein coupling [249].

There are two physiologically significant isoforms of GABA\textsubscript{B1} subunit, GABA\textsubscript{B1a} and GABA\textsubscript{B1b} that differ in N-terminal region. Expression of these two subunits is developmentally regulated at the transcription level. They are transcribed from the same gene after activation of alternative promoters [250]. Accordingly, there are two major subtypes of GABA\textsubscript{B} receptor, one formed from GABA\textsubscript{B2} and GABA\textsubscript{B1a} subunits, and the other formed from GABA\textsubscript{B2} and GABA\textsubscript{B1b} subunits [240].

GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits are structurally homologous and both possess two main domains: a heptahelical membrane domain that is responsible for recognition and activation of G-proteins (HD domain), and a large extracellular “Venus flytrap” domain (VFT domain) involved in ligand binding. Both domains oscillate between different conformational states, and these allosteric transitions are essential for receptor function and offer numerous possibilities for the allosteric regulation of receptor activity. GABA and other agonists bind
exclusively at the VFT domain of the GABA\textsubscript{B1} subunit, but VFT domain of the GABA\textsubscript{B2} subunit is necessary for the activation of whole receptor. On the other hand, GABA\textsubscript{B2} HD contains molecular determinants required for G-protein coupling, and by trans-activation mechanism, binding of GABA at VFT domain of GABA\textsubscript{B1} subunit lead to activation of HD domain of GABA\textsubscript{B2} subunit [251-253]. However, the HD of GABA\textsubscript{B1} improves coupling efficacy. Conversely, although GABA\textsubscript{B1} extracellular domain is sufficient to bind GABA\textsubscript{B} ligands, the extracellular domain of GABA\textsubscript{B2} increases the agonist affinity on GABA\textsubscript{B1}, and is necessary for agonist activation of the receptor. Altogether, this indicates that multiple allosteric interactions between the two subunits are required for wild-type functioning of the GABA\textsubscript{B} receptor [251].

Geng et al. [254] presented the crystal structures of heterodimeric GABA\textsubscript{B} complex consisting of extracellular VFT domains of GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits in the apo, agonist-bound and antagonist-bound forms. The apo and antagonist-bound structures represent the resting state of the receptor; while the agonist-bound complex corresponds to the active state. They found that both subunits adopt an open conformation at rest, but only GABA\textsubscript{B1} VFT closes on agonist-induced receptor activation. Furthermore, they revealed a unique activation mechanism for GABA\textsubscript{B} receptor that involves the formation of a novel heterodimer interface between subunits.

GABA\textsubscript{B} receptors are not modulated by benzodiazepines, barbiturates, or steroids, and are not sensitive to bicuculline [218,225]. Characteristic agonists of GABA\textsubscript{B} receptors are baclofen, a lipophilic derivative of GABA, and 3-aminopropylphosponous acid (3-APPA; CGP27492), while saclofen, phaclofen and 2-hydroxysaclofen act as antagonists of GABA\textsubscript{B} receptors [12]. Baclofen was introduced to the market in 1972 and is used to treat spasticity and skeletal muscle rigidity in patients with spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, and cerebral palsy. However, although GABA\textsubscript{B} agonists showed promising therapeutic effects in a whole range of other indications, they exhibit numerous side effects, including sedation, tolerance, and muscle relaxation [237,256].

Conclusions

GABA\textsubscript{A} receptors mediate most of the fast synaptic inhibition in the mammalian brain and are targeted by many clinically important drugs. They are subject to modulation at a variety of allosteric sites, with pharmacology dependent on receptor subunit combination. In addition to GABA, naturally occurring steroids can potently and specifically enhance GABA\textsubscript{A} receptor function in a direct manner, and consequently exert anxiolytic, analgesic,
anticonvulsant, sedative, hypnotic and anesthetic properties. GABA receptors are thus important for function and plasticity of the CNS. Analysis of the specific roles of GABAAR subtypes reveals their involvement in the pathophysiology of major CNS disorders, and opens novel perspectives for therapeutic intervention. Further pharmacological studies will contribute to more complete understanding of numerous interactions between various ligands and their binding sites on GABA receptors that might improve current pharmacological approach in treating various diseases.

Acknowledgments

This work was supported by Croatian Ministry of Science, Education and Sports to MJJ and JV.

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