Trends in Pharmacological Sciences

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REVIEWS

Review

The Benzodiazepine Binding Sites of $GABA_A$ Receptors

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Everyday activity is based on a subtle equilibrium of excitatory and inhibitory neuronal systems. The most prominent players in neuronal inhibition are synaptic and extrasynaptic $GABA_A$ receptors. Benzodiazepines are popular drugs that act as positive allosteric modulators of a subset of these receptors. Benzodiazepines have sedative, hypnotic, muscle-relaxant, and anticonvulsive effects, and are of outstandingly low overdose risk. The discovery of a large number of subtypes of $GABA_A$ receptors has raised hopes for a clear separation of this spectrum of actions. We discuss here how far this separation has been achieved, and outline recent progress towards the discovery of novel ligands for canonical and non-canonical binding sites.

Highlights

Multiple non-canonical sites of benzodiazepine actions including structurally non-homologous sites in the transmembrane domain have been described.

Extrasynaptic $GABA_A$ receptors responsive to ligands of the benzodiazepine chemotype are valuable drug targets.

Pharmacogenetic approaches lead to disentangling of the physiological function of individual receptor subunits.

Negative allosteric modulators acting selectively at specific benzodiazepine binding sites may, contrary to expectations, represent useful drugs, as exemplified by basmisanil.

Transgenic animals point the way towards potentially novel therapeutic indications of a new generation of benzodiazepines with improved selectivity.

Optogenetic methods have started to be applied to $GABA_A$ receptor research and may contribute useful insights.

Benzodiazepines Past, Present, and Future

Ligands of the high-affinity **benzodiazepine** (see Glossary) binding site have been immensely successful drugs for the past decades. Unlike many other drugs they are nearly free from acute and chronic toxicity [1]. For treatment of sleep disorders they are the drugs of choice, and they are often used in anxiety disorders. Additional popular uses are as sedatives in anesthesia and in psychiatric use, as well as centrally acting muscle relaxants. Why should they still be of scientific interest 57 years after their introduction to the market? Does this type of drug have a future? This review explains why the answer is still yes. Benzodiazepines exert their effects through **$GABA_A$ receptors** that respond to the neurotransmitter **GABA**. Benzodiazepine site-targeting drugs such as **diazepam** or **Z-drugs** (Box 1) bind with high affinity to some subtypes of $GABA_A$ receptors ([2,3] for review) and with low affinity to different sites in most other receptor subtypes. Understanding these receptor subtypes offers abundant opportunities for the separation of the pleiotropic effects of benzodiazepines that include sedation, hypnosis, anxiolysis, and muscle relaxation. At present, novel applications for benzodiazepines in neuropsychiatry are being investigated, for example in cognitive enhancement, pain relief, and possibly in the treatment of depression [4,5]. Moreover, ligands of high-affinity benzodiazepine binding sites are valuable tracer molecules for ligand-based imaging methods such as positron emission tomography (PET) [6] and single-photon emission computed tomography (SPECT). Recent developments in ligand discovery, binding-site characterization, and mapping of pharmacological effects to specific receptor subtypes are reviewed here. We first discuss the molecular properties of $GABA_A$ receptors.

$GABA_A$ Receptors Are Sites of Action of Benzodiazepines

The high-affinity binding site for benzodiazepines was first isolated from bovine brain and was described as a protein complex with two subunits named α and β [7]. This protein complex turned out to be identical to the $GABA_A$ receptor. Cloning and expression of these two subunits confirmed the identity of the protein complex [8]. It was rapidly recognized that there are multiple subunit isoforms, and to date subunits named α1–6, β1–3, γ1–3, ρ1–3, δ, ε, θ, and π have been identified in mammalian species ([9–11] for review) (Box 2). One to five out of these

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subunit isoforms are assembled into a ring-like pentameric complex with a central chloride and bicarbonate ion-selective channel. Binding of the neurotransmitter GABA opens this channel, and benzodiazepines modulate this opening. The major subunit isoform consists of α1, β2, and γ2, arranged α1γ2β2α1β2 counterclockwise as seen from the outside of the cell [12–14]. The two binding sites for the agonist GABA are located at the extracellular subunit interfaces β2/α1, and the high-affinity binding site for benzodiazepines is located at a closely homologous position at the α1/γ2 interface in this receptor. There may be dozens of other $GABA_A$ receptor subtypes (Boxes 2,3). Even receptors with the same subunit composition may assemble into different receptor species [15]. It has been shown that different subcellular compartments of neurons, such as postsynaptic densities, perisynaptic dendritic regions, and cell somata, contain distinct receptor subtypes [16].

Receptors containing an α_x/γ_y subunit interface, where x = 1, 2, 3, 5 and y = 1–3, form a high-affinity binding site for benzodiazepines (Figure 1A), for the later developed Z-drugs of different chemotypes, and for a large number of chemically distinct research compounds [17,18] (Box 1 for examples). For receptors containing the γ1 or the γ3 subunits, the affinity and efficacy of many ligands are reduced [19–21]. The α4 and α6 subunits provide a binding site for only a limited selection of compounds, and thus have traditionally been termed diazepam-insensitive (or DI) subunits to distinguish them from the diazepam-sensitive (DS) subunits [22]. Similarly, many of the less-abundant $GABA_A$ receptor isoforms do not bind benzodiazepines with high affinity ([11] for review).

The majority of $GABA_A$ receptors are expressed in neuronal tissues, but the receptors are also found in many other organs. Therapeutic potential of receptors in airway smooth muscle to alleviate spasms in asthma bronchialis is currently being explored [23,24]. However, we concentrate here on neuronal receptors. Synaptically located $GABA_A$ receptors, that are thought to mainly be composed of receptors assembled from two α, two β, and one γ subunit(s), mediate chloride ion currents at a single synapse that last for milliseconds. Another class, termed extrasynaptic receptors, mediate chloride currents lasting minutes and hours in large parts of the entire neuron, thereby adjusting excitability of the cell [25].

Although this review focuses on modulation of $GABA_A$ receptors by ligands of benzodiazepine binding sites, it should be pointed out that these receptors also respond to a wide variety of other modulators, including diverse toxins, barbiturates, intravenous anesthetics such as propofol and etomidate, volatile anesthetics such as isoflurane, neurosteroids, insecticides, and plant compounds (reviewed in [26]).

Structure of $GABA_A$ Receptors

The $GABA_A$ receptors belong to the family of **cys-loop receptors**. Insight into their atomic-level structure was provided by crystal structures of homologous proteins. First, the acetylcholine binding protein from *lymnaea stagnalis* [27] was analyzed, followed by pentameric

Box 1. Ligands of the Benzodiazepine Binding Sites

While the first 'benzodiazepine drugs' were 1,4-benzodiazepines (such as diazepam, Figure I), many ligands of the high-affinity benzodiazepine binding site that were subsequently developed have a non-benzodiazepine structure. Among the most prominent chemotypes are β-carbolines (such as DMCM and abecarnil), the heterogeneous group of Z-drugs (such as zolpidem, zaleplon, and zopiclone), and pyrazoloquinolinones (such as CGS 8216). Most chemotypes comprise positive allosteric modulators, null (or silent) modulators, and negative allosteric modulators (Box 3). Unselective positive allosteric modulators are anticonvulsant, sedative-hypnotic, and anxiolytic, while unselective negative allosteric modulators are pro-convulsant and anxiogenic. Null or silent modulators antagonize these effects *in vivo*, such as the benzodiazepine antidote flumazenil.

Glossary

Allosteric modulator: a molecule that binds to a site distinct from that bound by agonists in proteins and that does not induce activity by itself, but modulates activity triggered by an agonist.
Benzodiazepine: a class of drugs that mainly affect the central nervous system. Classical benzodiazepines promote neuronal inhibition.
Cys-loop receptor: a family of homologous receptors composed of subunits that contain a characteristic sequence flanked by two cysteine residues.
Diazepam: one of the first benzodiazepines marketed under the name valium.
GABA: γ-amino butyric acid, the major inhibitory neurotransmitter. This is in fact a misnomer because only very small amounts of the acid form of the neurotransmitter are present at physiological pH.
$GABA_A$ receptor: a ligand-gated chloride-selective ion channel belonging to the cys-loop family of pentameric receptors.
***In silico*:** procedures carried out using computational methods.
Z-drug: ligands of the high-affinity benzodiazepine site that have a non-benzodiazepine structure; examples include zolpidem, zaleplon, and zopiclone.

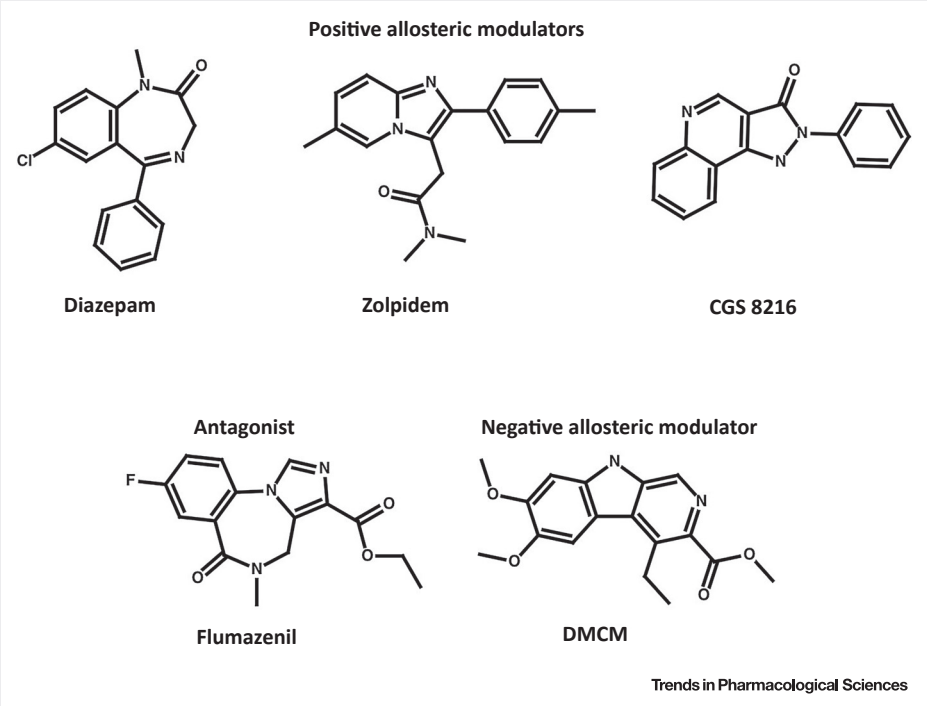

Figure I. Representative Benzodiazepine and Non-Benzodiazepine Ligands of the Canonical High-Affinity Benzodiazepine Binding Site. Zolpidem is a so-called Z-drug. CGS 8216 is an antagonist at the canonical site and a positive allosteric modulator at the α/β subunit interface.

ligand-gated channels from bacteria and *C. elegans* [28–30]. However, a high-resolution structure of the benzodiazepine binding site within the $GABA_A$ receptor is still missing. A human homomeric β3 $GABA_A$ receptor that lacks the intracellular domain has been crystallized [31], but unfortunately does not respond to either benzodiazepines or the natural agonist GABA. Owing to the high homology among subunits it is considered nevertheless to be a realistic model structure for the overall architecture of the binding site. All cys-loop receptor pentamers contain many cavities [32], whereby benzodiazepines have been shown to interact with multiple distinctive sites at extracellular and transmembrane localizations (Figure 1B).

Classification of Benzodiazepine Binding Sites

Historically, the sites at extracellular α+/γ− interfaces have been referred to as a single 'high-affinity benzodiazepine binding site', even though multiple $GABA_A$ receptor isoforms have such a site. This was justified because many ligands bind with very similar potency to most or all of these receptor isoforms. These high-affinity sites are formed by multiple discontinuous protein segments, termed 'loops A–G' [27] (Figure 1C).

There are additional extracellular sites that are homologous to the canonical site (Figure 1B). The α+/β− interface is structurally similar to the α+/γ− interface. Therefore, it is of little surprise that some ligands of the high-affinity benzodiazepine site can also occupy this homologous subunit interface [33]. These compounds (e.g., CGS 8216; Box 1) can act as antagonists at the α/γ subunit interface and as positive **allosteric modulators** at the α/β subunit interface, or

Box 2. Subtypes of the $GABA_A$ Receptor Family

It is generally accepted, with support from the bulk of the experimental evidence, that the majority of $GABA_A$ receptors in adult mammalian brain are pentamers with the subunit composition two α1, two β2 (or two β3), and one γ2 subunits, namely $(\alpha1)_2(\beta2 \text{ or } \beta3)_2(\gamma2)_1$. For a large fraction of receptors that contain a non-α1 α subunit isoform (i.e., any of α2 to α6), it was shown that they often also contain one of the other α isoforms, for example the α1α6βxγ2 and α1α6βxδ receptors in the cerebellum [49] or α1α2- and α2α3-containing receptors in spinal cord [68,69] Generally, for receptors containing any of the six α and any of the three γ isoforms, the general subunit stoichiometry is thought to be preserved, namely $(\alpha)_2(\beta)_2(\gamma)_1$. The arrangement of such receptors is α1γ2β2α1β2 counterclockwise (if viewed from the outside of the cell) which has been verified experimentally for α1β(2 or 3)γ2 receptors (Figure 1). Receptors containing γ1 or γ3 subunits are thought to be arranged as those containing the γ2 subunit.

The composition and arrangement of δ-containing receptors is less clear and has been controversially debated. The δ subunit has been shown to coassemble with α4, α6 and α1 subunits, along with any β. The majority of authors favor an $(\alpha)_2(\beta)_2(\delta)_1$ stoichiometry, but the arrangement is less well established, and different groups using different techniques (e.g., atomic force microscopy, photo-affinity labeling of neighboring subunits, concatenated recombinant receptors) have proposed different arrangements and potentially promiscuous subunit assembly [70,71]. Isoforms of the β subunit also often occur in combinations of two different isoforms in one receptor, thus introducing more uncertainty about the precise arrangements of subunits that occur in native receptors. This is relevant for drug development because the subunit interfaces harbor binding sites at which specific receptors can be targeted selectively, or where binding should be avoided to reduce side effects.

The ρ subunits are thought to coassemble mainly with each other (i.e., the group of the previously termed $GABA_C$ receptors); however, coassembly with other subunits has also been suggested [72,73]. The composition and thus also stoichiometry and arrangement of ε- or θ-containing receptors remains unknown. Coassembly of either of the two subunits with α3 has been suggested based on colocalization in specific brain regions [74]. The π subunit is expressed mostly in non-neuronal cells, often together with β3, and it is not known in which pentameric assemblies it participates.

vice versa [34]. An additional high-affinity site for diazepam has recently been located in a homologous position at the β2+/γ2− subunit interface [35], which does not occur in αβγ receptors.

Furthermore, low-affinity sites that share no structural homology with the aforementioned sites have been described in the transmembrane domain. It has been discovered that, in α1β2γ2 receptors, potentiation of GABA-activated currents by high concentrations of diazepam is biphasic, with a high- and a low-affinity component [36]. Combined mutation of the homologous residues α1S269, β2N265, and γ2S280 in the second transmembrane domains (M2) each to isoleucine abolished this micromolar component of potentiation while the high-affinity component remained unaffected [36]. These residues are part of cavities homologous to the cavity occupied by ivermectin in the crystallized *C. elegans* GluCl receptors [30]. The

Box 3. Nomenclature

(i) α1β2γ2 $GABA_A$ receptors (and any other receptor containing the α1, 2, 3 or 5 subunits) have also been termed 'α1 receptors', 'α2 receptors' etc. Because the major forms of $GABA_A$ receptors consist of five subunits, often with two different α subunit isoforms in the pentamer, such abbreviations should be avoided; according to the nomenclature recommended by the International Union of Basic and Clinical Pharmacology (IUPHAR), all subunits present in a receptor should be specified. Only the α subunit neighboring the γ subunit defines the properties of the high-affinity site for benzodiazepines.

(ii) Classical benzodiazepines act as positive allosteric modulators by binding to a high-affinity binding site present in certain types of $GABA_A$ receptor. These compounds have also been termed 'agonists' even if they have little if any agonistic action on $GABA_A$ receptors. Historically, the full term was 'benzodiazepine receptor agonist' and refers only to 'agonistic' effects at the benzodiazepine binding site. If the high-affinity binding site for benzodiazepines is occupied by a compound that fails to affect the response to GABA, it is called an antagonist. This type of molecule has also been termed a null or silent modulator. If the site is occupied by a compound that leads to a decrease in the response to GABA, it is called a negative allosteric modulator. The latter class of compound has also been termed 'inverse agonists'.

homo-pentameric GluCl receptor harbors five identical ivermectin sites, one at each subunit interface. In $\alpha1\beta\gamma2$ $GABA_A$ receptors there are four different interfaces, each harboring the corresponding cavity (Figure 1B). At least three different, if not all, interfaces of $\alpha1\beta\gamma2$ receptors may bind diazepam [37]. Additional ligands of this site have been identified [38,39]. It is important to note that the presence of the non-canonical sites is not limited to $\alpha\beta\gamma$ receptors, and δ subunit-containing receptors are also modulated via these sites [39,40].

How Do Benzodiazepines Act at the Molecular Level?

Ligands of the high-affinity site include positive allosteric modulators, negative allosteric modulators, and antagonists (Box 3 for nomenclature). The presence of positive allosteric modulators induces a shift in the GABA concentration–response curve to lower concentrations. Conversely, negative allosteric modulators shift it to higher GABA concentrations. Positive allosteric modulators shift the equilibrium between the ligand-bound resting and pre-activated states before channel opening [41], without affecting the maximal current amplitude elicited by GABA. As detected in agonist ligand-binding studies, this is paralleled by increased agonist affinity. Benzodiazepines affect channel opening of $GABA_A$ receptors induced by either agonist binding site [42]. For ligands acting at non-canonical sites a detailed analysis is lacking. At the macroscopic level, such ligands also act as positive allosteric modulators, negative allosteric modulators, or antagonists [37].

Benzodiazepine site 'antagonists' are being used as benzodiazepine antidotes, and ideally are null modulators – in other words they bind silently without any enhancement or reduction of GABA-elicited charge transfer. However, a perfect null modulator has not yet been described. Many compounds that act as benzodiazepine antagonists *in vivo* have been shown to modulate at least some receptor isoforms under such experimental conditions that enable the investigation of defined receptors. For example, the clinically used benzodiazepine antidote flumazenil is a (weak) partial negative allosteric modulator. Its action is concentration-dependent. At low concentrations it acts as a weak negative allosteric modulator, and at 1 μM flumazenil is an antagonist for receptors expressed in *Xenopus* oocytes. At higher concentrations flumazenil acts as a weak positive allosteric modulator [43].

Occupancy of allosteric sites can promote the open, desensitized, or closed states of the receptors. Consequently ligands act as positive or negative allosteric modulators. $GABA_A$ receptors exist in at least one closed, one ligand-bound pre-activated, one open, and one desensitized conformation [44]. Each of these assumes additional different conformations in the presence of positive and negative allosteric modulators or antagonists of the benzodiazepine binding site. Thus, insight into the complexity of allosteric modulation cannot be gained from crystallographic studies alone because crystallographic structures represent static conformations that might not correspond to any of the physiological conformations.

Receptor Subtypes and Their Function – Critical Appraisal

Largely unselective ligands of the benzodiazepine binding site, such as diazepam, are known to elicit a wide range of *in vivo* effects including hypnosis, sedation, anxiolysis, and muscle relaxation. Genetically modified mice were introduced in an effort to attribute individual effects to receptors that contain specific α subunit isoforms [45]. Sedative effects were proposed to be mediated specifically by 'α_1 receptors' (Box 3 for preferred nomenclature). Attempts were made to separate sedative from anxiolytic effects, for example by reducing or eliminating action at 'α_1 receptors' ('$\alpha1$-sparing drugs'). It has been pointed out, however, that non-sedating anxiolytics are not generally $\alpha1$-sparing in functional assays using recombinant systems [46].

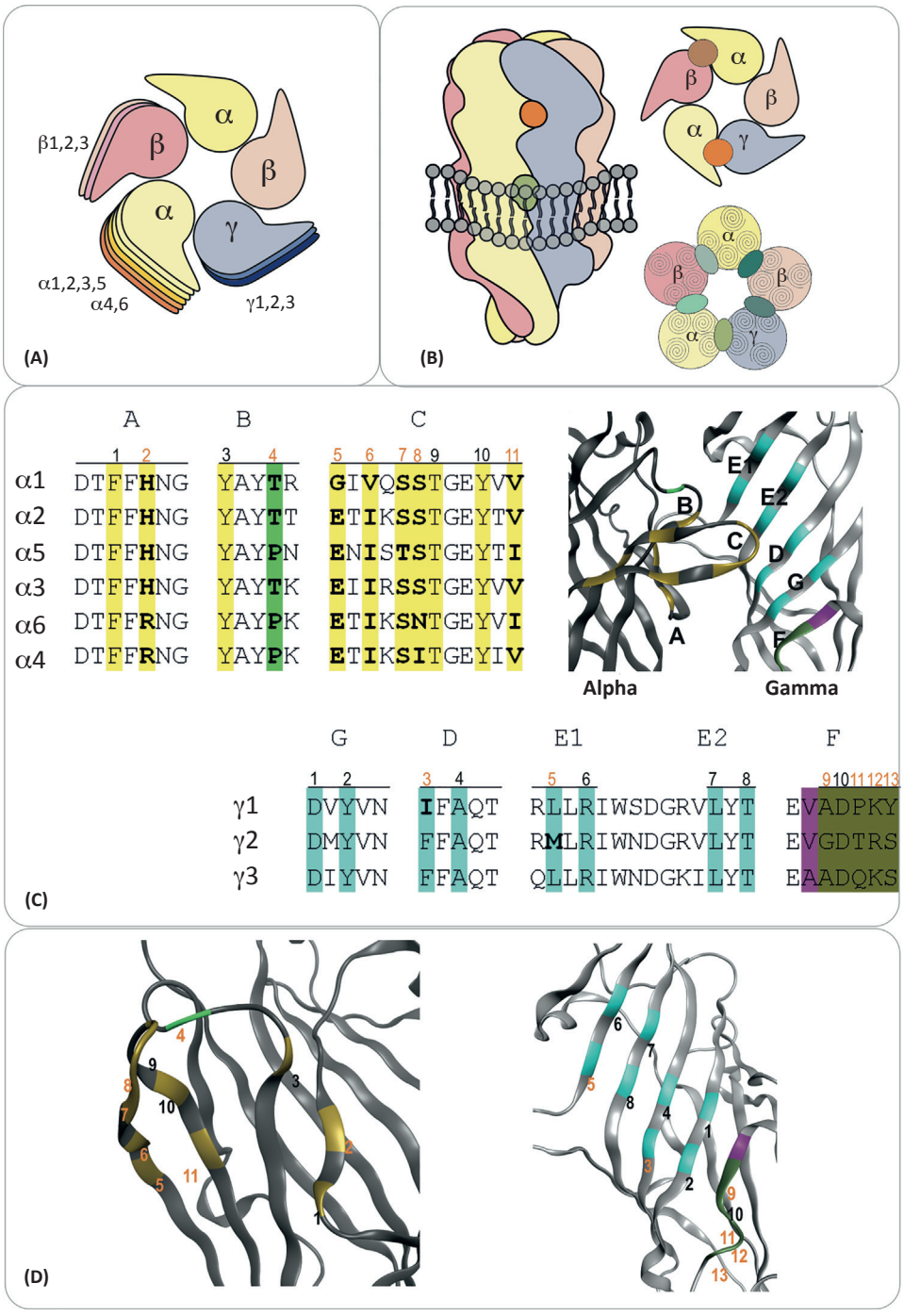

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Figure 1. Schematic Representation of a $GABA_A$ Receptor: Subunit Arrangement, Benzodiazepine Binding Cavities, and Architecture of the Canonical Benzodiazepine Binding Site. (A) A pentameric receptor may contain two different α and β subunit isoforms in addition to γ. If an α4 or α6 subunit neighbors the γ subunit, the receptors are diazepam-insensitive. (B) (Left) The $GABA_A$ receptor is shown embedded in the membrane with the classical high-affinity benzodiazepine binding site at the extracellular α/γ subunit interface (orange). The low-affinity site in the transmembrane

(See figure legend on the bottom of the next page.)

Other lines of research in fact correlated β isoforms with sedative-like, ataxic, or narcosis-inducing drug efficacy [47,48].

Recent technological advances now enable approaches orthogonal to transgenic animals with drug-insensitive subunits. Optogenetic manipulations have been established that allow light-mediated control of specific receptor populations, and will further help to map their physiological roles [49]. However, this novel approach to delineating the physiological role of defined receptor subtypes requires knowledge about subunit composition and arrangement of individual receptor species that is still largely lacking (Box 2).

Given that a large fraction of $GABA_A$ receptors contain two different α or β isoforms in a single receptor pentamer, it would be surprising if a single subunit that is expressed in many different receptor isoforms and in different cell populations could account for a single behavioral effect of any drug. As an illustration of the complexity, we discuss here the case of cerebellar granule cells (CGCs). As stated above, the high-affinity binding site for benzodiazepines has been located to the α*x*/γ subunit interface where *x* = 1, 2, 3, 5, but not 4 or 6. Many cells express multiple subunit isoforms. The CGCs express many subunits, among them α1, α6, β*x*, and γ2. It has been shown that α1α6β*x*γ2 receptors dominate over α6βxγ2 receptors [50]. Work in recombinant systems indicates that α1γ2β2α1β2, α6γ2β2α6β2, α1γ2β2α6β2, and α6γ2β2α1β2 receptors all differ in their functional properties and drug sensitivities. Only receptor assemblies with an α1 subunit adjacent to γ2 are responsive to benzodiazepines [15]. How does the CGC, or any other cell, assemble receptors, and which mechanisms lead to assembly of specific pentameric arrangements? Is receptor assembly dynamic? Can drug sensitivity thereby be modulated? All these questions are still open for research. Intriguingly, modulation of receptor localization and recruitment to synaptic versus extrasynaptic sites by benzodiazepines has been shown recently to occur in a bidirectional fashion. However, the mechanism of this relocation remain to be elucidated [51].

Beyond efforts to separate the established desirable effects (such as anxiolysis) from concomitant effects (such as sedation in the context of daytime anxiolysis) by subtype-specific targeting, additional potential therapeutic concepts based on subtype-selective targeting are being investigated (partly reviewed in [52]). Among them are pain states, affective disorders such as depression, and cognitive deficits in schizophrenia, developmental disorders, and neurodegenerative disorders (reviewed in [53]). In the treatment of depression, simultaneous prescription of antidepressants and benzodiazepines has involved off-label use of several benzodiazepines for some time, but the effectiveness of benzodiazepines in treatment of depression is probably limited to anxiolysis in cases where anxiety parallels depression [54,55]. Unfortunately, there remains an overall paucity of clinical studies that would permit systematic comparison of individual benzodiazepines in the different neuropsychiatric indications in which they are being heavily used off-label.

part of the receptor is also shown (green). A view from the outside of the cell with the classical high-affinity benzodiazepine binding site at the extracellular α/γ subunit interface (orange) and a homologous site at the α/β subunit interface (brown; top right). A section near the outer surface of the membrane showing that the low-affinity sites are present at all subunit interfaces (shades of green; bottom right). (C) Overview of the pocket and the partial alignment of the pocket-forming segments A–F. Pocket-forming positions of the α subunit are highlighted in yellow; the position that confers zolpidem sensitivity to the α1, α2, and α3 isoforms is highlighted in green. Pocket-forming positions of the γ subunit are highlighted in cyan, the conserved hydrophobic position that defines part of the loop F structure is highlighted in magenta, and putative pocket forming positions of loop F are highlighted in grey/green. Boldface letters indicate isoform-specific positions that impact on ligand selectivity. Orange numbers indicate all variable positions in the alignment (also shown in D). (D) Subunits, α (left panel) and γ (right panel) that form the pocket, viewed from the perspective of the ligand.

Recent progress has been made in translating animal findings on memory performance and amnestic effects – that are largely determined by tonically active receptors in which α5 contributes to the benzodiazepine binding site. Based on the observation that deletion of this subunit led to improved spatial learning performance [56], it was hypothesized that amnestic benzodiazepine effects (also seen in sedative anesthetics) can be attributed to excessive activity of this receptor population, and that nootropic effects can be elicited by α5-selective negative allosteric modulators [57]. This notion was confirmed in animal studies using several experimental compounds [58–60]. This line of research produced the so far furthest developed α5-selective negative modulator basmisanil (RG1662). The compound was tested in a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02024789) in a Down syndrome cohort for its ability to alleviate cognitive disabilities. No benefit was noted, but the compound may improve cognition in healthy subjects.

Cognitive deficits also occur as negative symptoms in schizophrenic patients, and benzodiazepines have a long history as candidates for this indication as well. A small-scale trial with bretazenil was terminated owing to excessive sedative effects despite promising efficacy [61]. α2 and α5 subunit-containing receptors were traditionally considered as the most promising targets to address schizophrenia symptoms [62,63]. At time of writing, basmisanil is being tested in a cohort of schizophrenic patients as an add-on for antipsychotic therapy to test whether negative symptoms can be reduced (https://clinicaltrials.gov/ct2/show/NCT02953639).

Overall, subtype selective targeting has been developed with success in rodent models. Proof of concept in humans is also well established for some receptor species – such as α5 subunit-containing receptors as targets for nootropic or amnestic effects. Translation of animal findings to human remains challenging, and trial failures may result from substantial sequence differences in several receptor subunits that impact on drug effects, as well as from differences in the expression patterns of individual subunits in distinct anatomical and cellular environments [64]. Moreover, individual receptor subtype composition and arrangement may also not be identical in different species for all receptor subtypes.

Novel Ligands for the High-Affinity Benzodiazepine Binding Sites

The development of benzodiazepines was traditionally based on large ligand series and experimental exploration of (quantitative) structure–activity relationships. There still is a paucity of selective compounds, and this nowadays could potentially be addressed by structure-guided ligand design. Box 4 reviews principles of structure-guided drug design. Computational models based on available homologous proteins have been in overall agreement regarding the relative positions of individual protein segments in the binding site [17]. Based on early mutational work, these segments were originally termed 'loops' A–G, with some being loops in the structural sense while others are short pieces of β strands [27,31]. The use of such homology models, in combination with a chemical biology approach to identify residues in contact with diazepam, has allowed relative positioning of diazepam into homology structures of an α1γ2β2α1β2 $GABA_A$ receptor. Virtual ligand screening into this structure has led to the discovery of novel ligands with high affinity for the canonical benzodiazepine binding site [38], thus validating the overall correctness of the structural models.

Owing to the high conservation of loops A–G in individual subunits, such as α2 and α3, or γ2 and γ3, the overall architecture of the individual sites is similar in different receptor isoforms. Only a few amino acids in the binding site confer unique properties to the pocket to enable ligand selectivity (Figure 1C,D). Owing to methodological limitations, models of highly

Box 4. Principles of Structure-Guided Drug Design

With reliable structural models of binding pockets, structure-guided drug design is possible. Based on an atomic model of the binding site, multiple structures help to explore factors such as locally variable regions and different pocket conformations. If these are not accessible, approximations can be obtained from computational predictions. For benzodiazepine sites the process is currently limited to homology models. A limitation is that the known protein structure imposes excessive similarity on the model structure. This will be the case if an α subunit is modeled from the available β subunit structure.

In principle, the pocket without ligand can be exploited. However, a ligated pocket gives confidence that the ligand can be accommodated sterically, and that favorable interactions stabilize the bound state. On the other hand, ligand poses (of experimental or computational origin) require further validation and do not necessarily reflect the pharmacologically active structure. Indirect experimental evidence can provide information on ligand orientation in the pocket, such as proximity-accelerated covalent reactions between functionalized ligands and functionalized pockets [75]. This helps to validate bound-state models.

In silico screening of candidate compounds into representations of bound states can be accomplished by different means. In our hands, a pharmacophore-based approach using the LigandScout program [76] performed well in delivering novel high-affinity scaffolds [38]. The ligand–pocket complex is represented by an abstract pharmacophore model. The steric properties of the pocket can be represented such that less-reliable or flexible pocket parts do not impose excessive constraints on the matching of molecules to the pharmacophore. Interactions between the reference ligand(s) and the pocket are described as abstract 'pharmacophore features' with defined positions and can include hydrophobic moieties, aromatic interactions, cation–π interactions, hydrogen bond-accepting and -donating features, and charged interactions (salt bridges). It is good practice to use pharmacophore models derived from several known binders to account for alternative usage of interaction valences in the pocket. Some algorithms also allow the inclusion of hydrogen bond-mediating water molecules that can occur as essential water molecules in bound states.

While structure-based *in silico* drug discovery algorithms will generate, in addition to true hits, a range of false positive and false negative findings, they still provide significant savings costs by limiting experimental screening to *in silico* hits instead of requiring large libraries. Moreover, model structures can be tested experimentally (e.g., mutational analysis). Clear benefits are the pre-selection of molecules for downstream experimental testing, and the testable structural hypothesis for further work.

homologous proteins (such as α2 and α3) based on a more distant family member (such as β3) will not reliably predict the subtle differences between individual isoforms of the high-affinity benzodiazepine binding sites.

The canonical binding sites to which α2, α3, or α5 contribute are highly similar. Therefore, differences in ligand affinity will not be large even if a ligand makes optimal use of the small differences in the pockets. As a possible alternative approach to achieve separation of compound effects, ligands with similar binding affinity but different allosteric effects at different receptor subtypes (functionally selective ligands) would be desirable. Ideally, a functionally selective ligand should bind silently (antagonist-like) at all subtypes except for one, where it should exert positive or negative modulatory effects. While this concept has led to some promising results [65,66], it remains to be explored whether 'silent' binding is indeed physiologically silent and will not lead to unwanted long-term adaptive changes in the nervous system. Moreover, structure-guided development of such ligands is made difficult for two reasons: (i) functional assays are much more time-consuming than binding assays, and (ii) structures of the benzodiazepine binding site in the apo- and the positively allosteric stimulated states are not known.

Non-Canonical Binding Sites May Be Interesting Targets

As outlined above, in addition to the high-affinity site for benzodiazepines, several non-canonical sites are present at subunit interfaces and in the transmembrane part of the receptor. Owing to the high similarity, for example between γ2 and γ3, or between α2 and α3, their high-affinity benzodiazepine binding sites are not suitable for reliable selective targeting, nor for binding or functional selectivity. The use of other allosteric binding sites might be a valuable

alternative. For the extracellular α+/β− binding sites, β1-selective ligands with potency in the nM range have already been reported [40].

The sites in the transmembrane domains generally are less well suited for subtype-selective ligands owing to the much higher sequence and structural conservation in this part of the subunits [33]. However, some specific subunits do feature unique pocket segments in this group of allosteric binding sites. For example, the α2 and α3 subunits, that have nearly identical extracellular plus sides (thus leading to very similar high-affinity benzodiazepine pharmacology), contain distinct M3 and M4 transmembrane segments [33] that can potentially be targeted individually by appropriate ligands.

So far, limited data (which are restricted to diazepam) make it difficult to predict in detail drug action via the non-canonical sites. Mice carrying a point mutation that renders the canonical binding site diazepam-insensitive in all four DS α subunits were studied [4]. These mice were protected from diazepam-induced muscle relaxation and motor impairment. These mice showed, under treatment, reduced locomotor activity that was relatively prominent at higher doses. This indicates that at least part of this residual response to diazepam could be mediated by non-canonical sites. It should be noted that minor effects may easily be missed in behavioral experiments.

Concluding Remarks

There are several major areas for future research. First, the exact composition and function of receptor isoforms must be investigated in more detail. While the biological effects of receptors that contain different α subunit isoforms are being gradually unraveled, it still is largely unclear into which receptor species (subtypes) they are assembled, or what the roles of the individual γ isoforms are in terms of receptor physiology and pharmacology. It is also not known whether homologous receptor species mediate homologous physiological functions in humans versus laboratory animals. To address some of these questions, exact receptor compositions and subunit arrangements need to be worked out for all receptor isoforms that mediate benzodiazepine effects (see Outstanding Questions).

Second, more clinical research would help to better understand and quantify differences between pharmacologically similar drugs, and would provide scientific evidence to the 'prescription practices' that often differ markedly between countries and even between institutions of the same country. Moreover, systematic clinical research would better delineate the most pressing medical needs that should be addressed in translational and basic research. Establishing the subtype preferences of clinically used benzodiazepines at defined, recombinantly expressed receptors containing canonical and non-canonical binding sites may help to close the gap between preclinical and clinical findings, and would allow mapping of drug binding sites to different desired and undesired benzodiazepine effects.

Third, rational design of drugs with better-defined subtype profiles is urgently needed. While an enormous number of small molecules from ~100 chemotypes have been identified as ligands of the canonical benzodiazepine binding sites, for the majority of them the subtype profiles are only partially known. In days of high-throughput and big data science, a structured repository and standardized protocols for the determination of full electrophysiological characterization of compound effects on a large panel of receptor isoforms and their respective benzodiazepine binding sites should be feasible. Such data would then assist in identifying the most promising compounds for subsequent lead optimization to obtain ligands with novel or improved subtype preferences. ***In silico*** methods of drug discovery such as structure-based pharmacophore

Outstanding Questions

Given the 19 $GABA_A$ receptor subunit genes and higher number of subunit isoforms that result from additional splice isoforms, many receptor species are still uncharacterized beyond those that are routinely investigated in pharmacological studies. What is the subunit composition, arrangement, and function of the native $GABA_A$ receptors that so far have not been defined?

The availability of a photoswitchable ligand that could be excited at single synapses would allow the effects of drug modulation of this synapse to be investigated for network activity. If applied to living animals, the effects of drug modulation in a small brain area on animal behavior could be studied. Can such a photoswitchable ligand be developed?

What are the consequences of the different subcellular compartmentalization of $GABA_A$ receptor isoforms for drug actions?

Many environmental and pharmacological stimuli lead to changes in subunit expression. What are the mechanisms leading to possible plastic changes in the subunit composition, and therefore drug sensitivity, of native $GABA_A$ receptors?

Atomic models of binding sites can be exploited for structure-guided drug design. What are the precise structural differences in binding pockets for benzodiazepines in different receptor isoforms?

Can *in silico* drug discovery methods augmented by experimental data accelerate the quest for drugs with high subtype selectivity?

Is it possible to find high-affinity ligands for the non-canonical binding sites located at subunit interfaces outside and within the membrane that are interface-specific?

screening of libraries, augmented with experimental data [38], would further accelerate the development from lead compounds to useful research tools or even novel therapeutic drugs.

Fourth, non-canonical sites may be targeted. Classical benzodiazepines require the presence of a γ subunit for high-affinity binding, which limits their activity to a specific large pool of receptor isoforms, leaving other isoforms unaffected. In particular, δ subunit-containing receptors, as well as less-studied receptor populations such as θ or ε subunit-containing receptors, are thought to mediate very specific physiological functions and thus could be potentially interesting targets for novel therapeutic approaches [67]. Non-canonical benzodiazepine sites are present on a wide range of receptors because they can also be formed by α and β subunits. Given that the benzodiazepine scaffold exhibits a superbly benign toxicological profile and excellent ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, developing some degree of specificity for activity at non-canonical sites offers a very valuable avenue to explore novel medicinal chemistry on the background of established drugs.

The $GABA_A$ receptor and its drug binding sites may thus be predicted to play a prominent role in the search for treatment of central nervous system diseases.

Acknowledgments

We thank Dr V. Niggli for critical reading of the manuscript.

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