ELSEVIER

$GABA_A$ receptor subtypes: any clues to the mechanism of benzodiazepine dependence?

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Chronic use of benzodiazepines for the treatment of anxiety has revealed that these drugs can lead to dependence as indicated by withdrawal symptoms following cessation and tolerance to the drugs effects. Together with their reinforcing properties, this has led to them being labelled as scheduled drugs. Our new knowledge regarding the molecular structure of the benzodiazepine binding site and the growing ability to differentiate $GABA_A$ receptor subtypes, either by genetic manipulation or subtype selective compounds, have begun to facilitate our understanding of what underlies the mechanism of benzodiazepine dependence. In addition, the involvement of $GABA_A$ receptors in this phenomenon is leading to a greater understanding of other drugs such as alcohol and opiates.

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Abbreviations

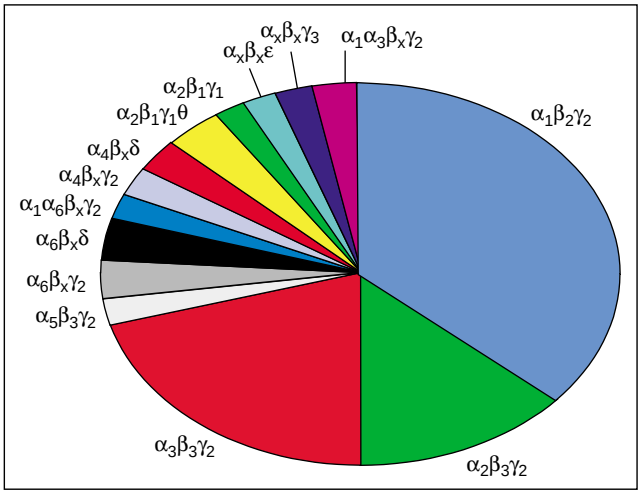
GABA γ-aminobutyric acid
NMDA N-methyl-D-aspartate

Introduction

Benzodiazepines have been prescribed by doctors since chlordiazepoxide (Librium) was first introduced in 1960. They are extremely effective anxiolytic agents as well as being useful for many other indications, including insomnia, convulsive disorders, muscle relaxation and sedation. Following Librium, many related compounds with varying potency and pharmacokinetic properties were developed and, because of their safety and efficacy, benzodiazepines became the most prescribed drugs in the 1960s and 1970s. However, it became apparent that there were several side effects that resulted from use of these drugs. The immediate drowsiness and confusion were clearly a problem for patients required to perform highly attentive tasks such as driving; however, it became obvious that a more serious problem occurred following long-term treatment, whereby patients could become both physically and psychologically dependent upon benzodiazepines, experiencing acute withdrawal phenomena after abrupt cessation of treatment. Benzodiazepines have also been classified as drugs of abuse, yet this is rarely in isolation, and 80% of benzodiazepine abuse is in conjunction with other abused drugs such as opiates and alcohol [1]. As a consequence, the use of benzodiazepines has fallen in recent years; however, they are still highly prescribed drugs and more stringent guidelines have been put in place so that they are generally prescribed for no longer than a one-month period. Clearly, a replacement compound that was as effective as a benzodiazepine but lacked these side effects would be in great demand, and pharmaceutical companies have long sought to find a way of tackling this problem. Initially, it was thought that a partial agonist would retain the anxiolytic efficacy of full benzodiazepine agonists but lack many of the side effects. Several compounds were generated to test this hypothesis, such as bretazenil and FG8205; however, these potential drugs were never developed owing to the persistence of the associated liabilities such as sedation.

Benzodiazepines act by enhancing the effect of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Furthermore, they bind directly to a site on the receptor, altering the functional response upon receptor activation by GABA. In 1987 the first $GABA_A$ receptor subunit was isolated and sequenced and shown to be part of a superfamily of ligand-gated ion channels including nicotinic acetycholine receptors and glycine receptors [2]. This family has become characteristically known as the 'cys-loop' family because of the presence of a cysteine loop in their N-terminal domain. All of these receptors exist as pentamers arranged around a central ion channel. Just like the cascade of benzodiazepines that followed chlordiazepoxide, more homologous sequences that could be classified as $GABA_A$ receptor subunits were isolated, until it became clear that this receptor was not a single entity but made up of many different subtypes, differing in the make-up of their constituent five subunits [3]. To date, 19 different subunits have been isolated not including alternatively spliced variants, which add further diversity. The five subunits are arranged to form a channel that is selectively permeable to chloride ions; GABA binds extracellularly to increase the probability of the channel opening. The flow of chloride ions hyperpolarizes the cell, decreasing the likelihood of the neuron firing an

Figure 1



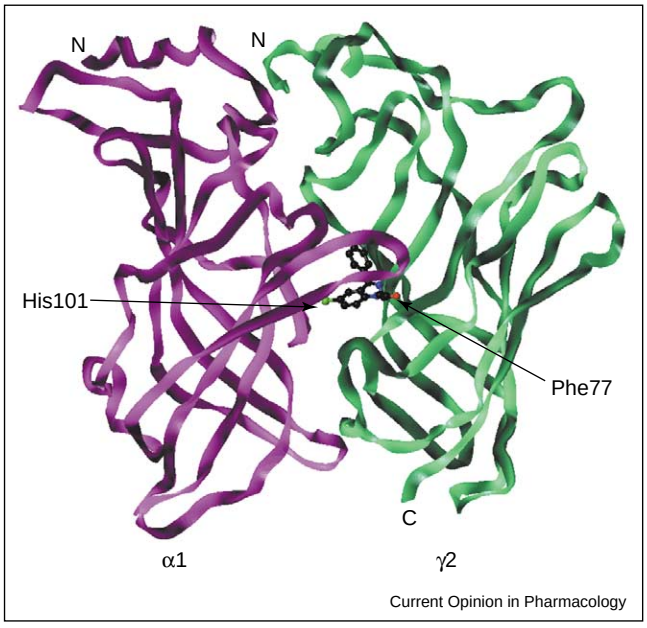
Pie chart illustrating the approximate abundance of different $GABA_A$ receptor subtypes in the mammalian brain. Subscript x is indicated where the particular subunit is not known. Reproduced with permission from [49].

action potential. The pentamer comprises of two α subunits, two β subunits and an additional subunit, most commonly γ2, which confers a key part of the required binding site for benzodiazepines. Subunits that have been isolated to date comprise α1–α6, β1–β3, γ1–γ3, δ, ε, θ, π and ρ1–3. Clearly from this number, the wealth of possible receptor subtypes is enormous; however, only a limited number have been shown to exist in reality. The most abundant combinations were demonstrated using immunolabelling and autoradiography, and are illustrated in Figure 1 [4]. These receptor subtypes have discrete distributions in the brain, suggesting that they fulfil different functional roles [5]. Pharmacological experiments on recombinant receptors have also revealed that these subtypes show distinct characteristics based on their responses to various ligands [6]. Those receptors containing a γ2 subunit possess a binding site for benzodiazepines, but those also containing an α4 or α6 subunit are not sensitive to the majority of clinically prescribed benzodiazepine agonists. The discovery that a family of $GABA_A$ receptors existed with distinct functional roles provided the opportunity to revisit the benzodiazepine dilemma, as it became clear that it might be possible to separate the beneficial anxiolytic effects from the side effects through pharmacological isolation of subtypes. Recent advances of our understanding of the structure of the benzodiazepine binding site and the ability to make either subunit knockout mice or mice expressing targeted mutations has created a superb opportunity to further investigate the roles played by receptor subtypes in the efficacy and side effects of benzodiazepine treatment [7,8]. Although in their early stages, these techniques are beginning to reveal valuable information on the mechanisms and receptor subtypes involved not only in benzodiazepine addiction but also in other forms of drug dependency.

$GABA_A$ receptor structure

As dependency and withdrawal phenomena are dependent upon chronic occupation of the benzodiazepine binding site, the understanding of this domain of the receptor is an important first step towards understanding the underlying mechanisms. As mentioned previously, the benzodiazepine binding site is located in the extracellular N-terminal portion of the receptor. Both the α subunit and the γ subunit contribute to the binding site and mutagenesis studies have implicated several residues or groups of residues in contributing to this binding pocket. A recent description of the structure of a molluscan acetylcholine binding protein has greatly facilitated our understanding of the structure of the extracellular region of the $GABA_A$ receptor, as the sequence of the molluscan protein closely matches the N-terminal region of all 'cys-loop' channels [9]. By overlaying the α and γ subunits onto the molecular model of the acetylcholine binding protein, the structure of the regions involved in the benzodiazepine binding site have been modelled, with previously identified amino acids clustering together to form a putative binding pocket (Figure 2) [10]. Several crucial amino acids are involved in benzodiazepine binding or function: α1His101, α1Tyr159, α1Gly200, α1Thr206 and α1Tyr209 in the α subunit, and γ2Phe77, γ2Ala79, γ2Thr81 and γ2Met130 in the γ2 subunit. In addition to site-directed mutagenesis, a technique known as 'substituted cysteine scanning' has been used to investigate these amino acids, whereby the residues of interest are mutated to cysteine that can then be covalently labelled by cysteine-reactive compounds such as methanethiosulfonate. Binding of a benzodiazepine will prevent the cysteine labeling, suggesting that the residue forms part of the binding site [11••,12]. In the α subunit, the His101 (or equivalent) is a key binding residue, with γ2Phe77 also being a major residue [13,14]. The two GABA binding sites per receptor are located in a homologous position to the benzodiazepine site between the α and β subunits. The mechanism of the ligand-binding to channel-opening process is still unclear; however, benzodiazepine agonist binding has no direct functional effect but, in the presence of GABA, increases the frequency of channel opening producing a net increase in current flow. Recent studies have provided clues as to what might be happening to the channel under these circumstances [15••] and molecular modelling should assist in developing our understanding of the process of ligand binding, channel opening and desensitization. Clearly, the diversity of receptor subtypes and their associated pharmacology complicate the search for the mechanism of dependence, but suggest that not all GABA receptors are involved.

Figure 2



Model of the extracellular domain of an α1 and γ2 subunit of the GABA$_A$ receptor based on the nicotinic acetylcholine binding protein. The model shows diazepam located in the binding site and highlights the position of His101 in α1 and Phe77 in the γ2 subunit.

GABA$_A$ receptors and dependence

Tolerance at the receptor level

It is well established that following chronic exposure to benzodiazepines and alcohol there are alterations in GABAergic neurotransmission. These alterations contribute to the symptoms of tolerance, dependence and withdrawal. The nature and mechanism of these changes are not clear; however, they are closely linked to the dose of benzodiazepine and the duration of use [16,17]. This area of the literature is dogged by a great deal of variability, but there are some findings that are reasonably consistent. Firstly, there appears to be a reduction in the potentiation of GABA responses by acute application of benzodiazepines following chronic benzodiazepine exposure, so that chronically treated receptors become less sensitive to an acute challenge. This effect is intrinsic to the receptor itself, as a cell line expressing one recombinant subtype (i.e. α1β2γ2) undergoes the same process [18,19]. The turnover of receptors is important in this process, as the effect is blocked by cyclohexamide [20].

Changes in GABA$_A$ subtype expression

It has also been observed that chronic benzodiazepine treatment produces alterations in the expression of individual subtypes. The evidence is somewhat confusing, with some reports describing upregulation of particular subunits [20], whereas others see either no effect [21,22] or downregulation [23]. It is likely that these changes are local to specific brain regions. In addition, the extent of changes in mRNA is often relatively small and might not reflect equivalent protein changes. A study investigating receptor protein levels demonstrated a decrease in α1 and upregulation of α3, α5, β2/3 and γ2 subunits following two weeks of exposure to diazepam [24]. In terms of the functional effects of chronic treatment, reduced miniature inhibitory synaptic current amplitude was reported recently in the hippocampus; this was associated with a decrease in α1 subunit mRNA and decreased protein kinase A activity [25]. A recent study used 2-deoxyglucose quantitative autoradiography to monitor changes in activity following diazepam treatment. They observed a reduced level of glucose utilisation on acute diazepam exposure, which tolerated over a 28-day period of daily treatment [22]. This study demonstrated short-term tolerance after three days in brain regions associated with sensory processing but a longer term effect in the Papez circuit, nucleus accumbens and basolateral amygdala, which are all involved in emotional processing, suggesting that these changes might more closely follow the development of dependence. Changes in these regions were also observed on withdrawal, implicating a common circuitry in the withdrawal process.

Involvement of GABA$_A$ receptors in other forms of dependence

The changes observed after chronic benzodiazepine treatment mimic, at least in part, those following chronic alcohol exposure and, as ethanol produces similar dependence and withdrawal properties and acts at least in part

through $GABA_A$ receptors, these studies might reveal common mechanisms. As with benzodiazepines, GABA subunit expression is altered following chronic ethanol administration. The α1 subunit is reduced and α4 increased in specific regions [26,27], and this is reflected in the pharmacology of hippocampal GABAergic function where miniature inhibitory synaptic currents become less sensitive to benzodiazepine potentiation [28]. Biochemical studies using coimmunoprecipitation of cortical $GABA_A$ receptors with clathrin and adaptin-α reported an increase in the proportion of α1 subunit protein in the cytosol, suggesting that α1 subunit endocytosis is enhanced [29••]. In that study, α4 peptide was also upregulated but there was no change in its cytosolic fraction. In addition, genetic analysis studies reveal that GABAergic genes are probably linked to alcohol dependence and withdrawal in mice [30] and, recently, the α2 and γ3 subunits have been associated with alcohol dependence in humans [31,32]. More details on alcohol dependence and human genetic studies can be found in the reviews by Wonnacott, Sidhpura and Balfour, and Mayer and Höllt (this issue). We do not yet understand the mechanisms that underlie these associations; however, it appears that GABA receptors may play a role in determining addictive behaviour itself. Laviolette *et al.* [33••] demonstrated that $GABA_A$ receptors in the ventral tegmental area serve as a switching mechanism in the dopamine reward pathway. Following opiate dependence, these receptors switch from inhibitory to excitatory signalling [33••]; however, the subtypes involved remain unexplored. Using congenic mouse strains, the first gene to be linked to alcohol and barbiturate (another $GABA_A$ receptor modulator) dependence and withdrawal has recently been identified. *Mpdz*, which is believed to regulate protein targeting and stabilization in membranes, has been linked with 5-hydroxytryptamine $5HT_2$ and $GABA_B$ receptors, and its expression genetically correlates with withdrawal severity [34••].

In addition to changes in GABAergic neurotransmission in benzodiazepine-dependent animals, there are also reports of modifications in excitatory transmission. N-methyl-D-aspartate receptor (NMDA) antagonists have been shown to prevent the development of sedative tolerance [35]. Increases in α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and NMDA receptor expression also occur upon withdrawal from diazepam [36,37]. A recent study has shown that this upregulation is a result of increased expression of the NR1 and NR2B NMDA receptor subunits (in this case, in the hippocampus) and that MK801 administration was able to block this upregulation [38]. Clearly, these are downstream adaptations in response to overstimulation of $GABA_A$ receptors that could underlie, at least in part, some of the dependence properties. They are almost certainly involved in the withdrawal responses observed following cessation of chronic treatment.

GABA knockout and mutant mice

The ability to explore the mechanisms underlying dependence has recently been facilitated by the generation of $GABA_A$ subunit knockout mice [39,40] and point mutant mice rendering a single subtype insensitive to benzodiazepines [7,41]. Chronic studies have not yet been performed using any of the subunit knockout mice, as the compensatory changes from other subunits make interpreting the data difficult. The generation of mice containing a mutated histidine residue in the α1 subunit (as described earlier), which renders the $GABA_A$ receptor insensitive to diazepam, offers a much more useful tool to investigate the mechanism of tolerance and dependence. The utility of these mice was first shown using the α1His101Arg mouse in which α1-containing receptors are diazepam insensitive. Wild-type and mutant animals were acutely treated with diazepam and 16 h later brain RNA was prepared for a microarray study to identify those genes regulated by diazepam in wild-type but not mutant animals [42••]. Several genes including those encoding calcium/calmodulin-dependent kinase II, brain-derived neurotrophic factor and mitogen-activated protein kinase were found to be downregulated only in association with α1. These types of study could potentially reveal a lot of information on gene regulation following chronic administration and more details on gene array experiments are discussed in the review by Rhodes and Crabbe (this issue). A study utilizing α1H101Arg, α2H101Arg, α3H126Arg and α5H105Arg has recently shown that the development of tolerance to the sedative properties of diazepam involves both α1 and α5 subunits. Because α1 animals were not sedated acutely, they did not develop tolerance. The α5H105Arg mice, despite being acutely sedated, also showed no tolerance. Wild-type mice exhibited reduced α5-associated binding in the hippocampus following chronic treatment [43••]. This finding supports that of a previous study showing downregulation of the α5 subunit [23], and the association of dependence with the α5 subunit reflects previous findings with the subunit-selective hypnotic zolpidem. Zolpidem does not bind to the α5-containing $GABA_A$ receptor and is reported to show less dependence liability than do non-selective benzodiazepines [44,45]; however, this has recently been questioned by baboon studies in which zolpidem was highly reinforcing [46]. Recent progress towards the development of subtype-selective agents has also been made with the discovery of benzodiazepine site agonists that selectively potentiate α3- and α2-containing receptors [47••,48]. These compounds lack the sedative profile of either non-selective or α1-selective agonists, but effects on dependence and withdrawal have not yet been published.

Conclusions

Despite significant literature on benzodiazepine dependence, including tolerance and withdrawal, we are still far from understanding what mechanisms underlie these

effects. The downstream consequences of long-term benzodiazepine occupancy of $GABA_A$ receptors can now be investigated in more detail using subtype-insensitive mice, and future work with these animals promises to reveal further insight. Large-scale microarray studies should also shed light on the gene regulation that is clearly involved in the adaptation process, and hopefully will facilitate the identification of the chain of events that lead to benzodiazepine dependence. The recent identification of subtype-selective compounds will also aid in identifying the types of $GABA_A$ receptor that mediate these phenomena.

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The utility of point mutant mice is again highlighted in this publication where α1, α2, α3 and α5 subunits have been rendered insensitive to diazepam and tolerance to the sedative activity of benzodiazepine treatment has been investigated. Clearly the lack of sedation in α1 mutated mice implicates this receptor subtype but, in addition, the authors have discovered that α5-containing receptors might also be important in conferring tolerance to the sedative properties of diazepam.

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