GABA<sub>A</sub> 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 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<sub>A</sub> 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<sub>A</sub> receptors in this phenomenon is leading to a greater understanding of other drugs such as alcohol and opiates.

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Current Opinion in Pharmacology 2005, 5:47–52

This review comes from a themed issue on Neurosciences Edited by Graeme Henderson, Hilary Little and Jenny Morton

Available online 24th November 2004

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DOI 10.1016/j.coph.2004.08.006

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<sub>A</sub> receptor subunit was isolated and sequenced and shown to be part of a superfamily of ligand-gated ion channels including nicotinic acetylcholine 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<sub>A</sub> 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
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 in benzodiazepine binding site between the α1–α6, β1–β3, γ1–γ3, δ, ε, θ, π and ρ1–3. 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 which 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.

**GABA<sub>A</sub> 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<sub>A</sub> 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 which 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.
GABA<sub>A</sub> 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. α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>) 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<sub>A</sub> 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 α<sub>1</sub> and upregulation of α<sub>3</sub>, α<sub>5</sub>, β<sub>2/3</sub> and γ<sub>2</sub> 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 α<sub>1</sub> 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<sub>A</sub> 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 Höltt (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 congeneric mouse strains, the first gene to be linked to alcohol and barbiturate (another GABA_A receptor modulator) dependence and withdrawal has recently been identified. Mptz, which is believed to regulate protein targeting and stabilization in membranes, has been linked with 5-hydroxytryptamine 5HT_1A and GABA_A 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 subtype-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<sub>A</sub> 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<sub>A</sub> receptor that mediate these phenomena.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


This paper has used the model proposed by the nicotinic acetylcholine binding protein to predict important residues involved in GABA<sub>A</sub> receptor channel activation. They show that electrostatic interactions between aspartate and lysine residues in the extracellular domain and in the transmembrane 2-3 linker domain are critical for determining channel gating. They suggest that these residues move closer together on channel opening, and is the first publication describing a possible mechanism for coupling ligand binding to channel activation.


28. Liang J, Cagetti E, Olsen RW, Spigelman I: Altered pharmacology of synaptic and extrasynaptic GABA<sub>A</sub> receptors on CA1
hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence. J Pharmacol Exp Ther 2004, 4:104.06798sv1-0.

29. Kumar S, Kralic JE, O’Buckley TK, Grobin AC, Morrow AL: Chronic ethanol consumption enhances internalization of alpha1 subunit-containing GABA<sub>A</sub> receptors in cerebral cortex. J Neurochem 2003, 86:700-708. This study explores in more detail the mechanism underlying the changes in subunit expression, in this case following chronic ethanol administration. They have focused on the downregulation of the alpha1 subunit in the synaptic membrane and demonstrate that this is caused by a change in trafficking producing a corresponding upregulation of alpha1 in the cytosol. They provide further evidence that endocytosis of the alpha1 subunit is enhanced on chronic alcohol administration.


33. Laviolette SR, Gallegos RA, Henrikse SJ, van der Kooy D: Opiate state controls bi-directional reward signaling via GABA<sub>A</sub> receptors in the ventral tegmental area. Nat Neurosci 2004, 7:160-169. Although not specifically addressing benzodiazepine dependence, this study highlights the important role played by GABA<sub>A</sub> receptors in the reinforcing effects of chronic opiate administration. They have focused on receptors in the ventral tegmental area, and show that, as the effects of opiate treatment switch from an acute dopamine-independent state to a chronic (opiate-dependent) dopamine-dependent state, the GABA<sub>A</sub> receptors switch from an inhibitory to an excitatory mode of action. They suggest that this GABA switch is a key molecular event defining the phase between acute drug versus dependent and withdrawn states of addiction.

34. Shirley RL, Walter NA, Reilly MT, Fehr C, Buck KJ: Mpdz is a quantitative trait gene for drug withdrawal seizures. Nat Neurosci 2004, 7:699-700. For many years the technique of quantitative trait loci analysis has been utilized to identify chromosomal regions involved in specific, such as in this case withdrawal from alcohol and barbiturate treatment. By developing congenic mouse strains, this study has enabled identification of the first quantitative trait gene associated with withdrawal. The Mpdz gene showed genotype-dependent differences in coding sequence, and the expression of Mpdz was associated with severity of withdrawal. This protein interacts with SHT<sub>3</sub> and GABA<sub>A</sub> receptors and future studies should reveal more about its involvement with receptor signaling.


38. Alimiron RS, Perez MF, Ramirez QA: MK-801 prevents the increased NMDA-NR1 and NR2B subunits mRNA expression observed in the hippocampus of rats tolerant to diazepam. Brain Res 2004, 1008:54-60.


42. Huopanieni L, Keist R, Randolph A, Certa U, Rudolph U: Diazepam-induced adaptive plasticity revealed by alpha1 GABA<sub>A</sub> receptor-specific expression profiling. Exp Ther Med 2009, 7:139-149. This study highlights the utility of microarray analysis in combination with animals with genetic mutations in specific subunits, in this case the alpha1H1s101 GABA<sub>A</sub> receptor subunit. Because the alpha1 subunit has been shown to mediate the sedative properties of benzodiazepines, a comparison of genes modified in wild-type and alpha1H1s101 mice in response to diazepam reveals putative downstream events associated with benzodiazepine activity at alpha1-containing receptors. This approach would be highly amenable to future chronic studies to determine genes associated with benzodiazepine tolerance, dependence and withdrawal.

43. van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohier H, Fritschy JM, Crestani F: Requirement of alpha5-GABA<sub>A</sub> receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci 2004, 24:6785-6790. The utility of point mutant mice is again highlighted in this publication where alpha1, alpha2, alpha3 and alpha5 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 alpha1 mutated mice implicates this receptor subtype but, in addition, the authors have discovered that alpha5-containing receptors might also be important in conferring tolerance to the sedative properties of diazepam.


47. Crawford J, Attack JR, Cook SM, Gibson KR, Nadin A, Owens AP, Pike A, Rowley M, Smith AJ, Sohal B et al.: Tricyclic pyridines as functionally selective human GABA<sub>A</sub> alpha2(3) receptor-ion channel ligands. Bioorg Med Chem Lett 2004, 14:1679-1682. The first description of a range of compounds which bind to the benzodiazepine site of the GABA<sub>A</sub> receptor and show selective potentiation of alpha2- and alpha3-containing receptors while acting as antagonists and being functionally silent at alpha1-containing receptors. These compounds will be excellent tools for differentiating the subtypes involved in conferring benzodiazepine dependence and withdrawal.
