GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery?

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The clinical importance of benzodiazepines, barbiturates and general anesthetics, all of which act through the γ-aminobutyric acid (GABA)-A neurotransmitter receptor, is testament to its significance as a CNS drug target. These drugs were all developed before there was any understanding of the diversity of this receptor gene family. Recent studies using genetically modified mice and GABA-A receptor-subtype-selective compounds have helped to delineate the function of some of these subtypes, and have revealed that it might be possible to develop a new generation of selective drugs with improved profiles or novel applications.

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Although the brain is undoubtedly the most wondrously complex organ, it is possible to distil the way it works into two opposing forces; excitation and inhibition. Neurons, using neurotransmitter receptor systems, are excited by the neurotransmitter glutamate, and inhibited by the neurotransmitter γ-aminobutyric acid (GABA) [1]. Over- or under-activity of either of these two systems has been proposed to have a role in a variety of neurodegenerative (e.g. stroke, Parkinson's disease, epilepsy [2,3]) or psychiatric (e.g. anxiety, schizophrenia [4]) conditions, which in some cases can be corrected by pharmacological intervention. A classic example is the benzodiazepine diazepam (perhaps better known as valium; 'mother's little helper' according to the Rolling Stones). Diazepam is a 'potentiator' (a positive allosteric modulator) of the neurotransmitter receptor through which GABA exerts its effects (the GABA-A receptor), acting through a binding site on the receptor distinct from the GABA binding site. There is no natural ligand for this allosteric binding site. Benzodiazepines like diazepam acts to pep-up the inhibitory activity and, thereby, simplistically, 'dampening' the anxiety felt by the patient. Similarly, in the case of epilepsy, diazepam acts to increase the inhibitory activity of neurons, thus reducing the excessive excitatory activity that occurs in certain brain regions during an epileptic seizure (discussed further below).

The GABA-A receptor: there is more than one

Although diazepam has been an extremely successful and widely prescribed anxiolytic drug, it is far from perfect. It is sedative (which is unacceptable if the goal is to treat anxiety in the context of continuing a normal life), has undesirable interactions if taken with alcohol, can be amnesic (although this can be considered advantageous in certain invasive and unpleasant investigative procedures, for example, colonoscopy, bronchoscopy), and can induce both tolerance and dependence [5]. How does diazepam mediate these effects and, if we understood this, could we develop a new generation of drugs that selectively targeted only the desired properties of benzodiazepines?

Insights into this first emerged in the late 1970s early 1980s, with the discovery that there were at least two distinct types of pharmacological binding sites for benzodiazepines in the brain (so-called BZ1 and BZ2) [6], and that the binding sites were on GABA-A receptors, through which benzodiazepines were most likely exerting their effects. With the application of molecular biology approaches, in the late 1980s and 1990s, it soon became clear that this was just the tip of the iceberg, and that a family of GABA-A receptor subtypes exists within the brain, formed by co-assembly from 16 different subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ; for a review see [7]). If all of these subunits could co-assemble to form a receptor molecule then the total number of potential receptor subtypes would be huge. Fortunately, and not surprisingly, nature has

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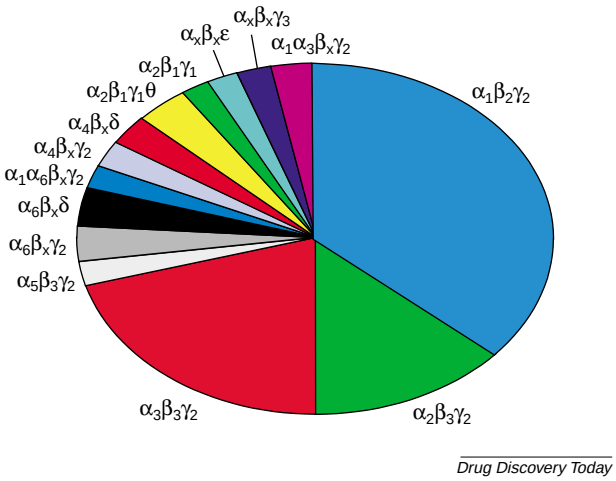

Figure 1. A pie chart representing the approximate abundance of γ-aminobutyric acid (GABA)-A receptor subtypes in the rat brain. Subscript x indicates the type of α, β or γ subunit is not known. This is a broad stroke representation, and other receptor subunit combinations probably exist.

instilled some order in this process; experiments using subunit specific antibodies to immunoprecipitate native receptor molecules and experiments expressing combinations of subunit cDNAs in mammalian cells have suggested that a more finite number of GABA-A receptor subtypes exists in the brain (Fig. 1) [7–9]. Perhaps even more encouragingly, from the perspective of drug discovery, only a subset of these GABA-A receptors were the site of action of benzodiazepines (for a review see [10]). Those containing α1, β and γ2 subunits corresponded to the BZ1 binding site mentioned previously, while those containing either α2, α3 or α5 in combination with β and γ2 subunits correspond broadly to the BZ2 binding site: a very satisfactory outcome.

The desirable and undesirable effects of benzodiazepines: which GABA-A receptor subtype does what?

With the insight that benzodiazepines, such as diazepam, most likely mediated their effects through binding, thereby allosterically potentiating a finite set of distinct GABA-A receptor subtypes, the crucial question was, 'do different receptor subtypes mediate the different clinical effects of benzodiazepines?' From the drug discovery perspective, the idealized outcome would be that different subtypes would be clearly responsible for the different effects of benzodiazepines; for example, the action of diazepam at one subtype would cause the sedation, the action at another would cause the anxiolytic (i.e. anti-anxiety) response, and so on. By contrast, a 'shades of grey' conclusion, where each receptor subtype contributed in part to each of the properties of benzodiazepines, would be disappointing. How can we answer these questions? The most obvious way is to use medicinal chemistry to develop a new generation of benzodiazepine site ligands that selectively bind or modulate only a single receptor subtype, and then simply determine their effects in animal models. However, this is an enormous undertaking! A somewhat more tractable approach became possible by returning to another earlier observation that benzodiazepines, such as diazepam, bound to α1βγ2, α2βγ2, α3βγ2 and α5βγ2-containing receptors with high affinity, but α4βγ2 and α6βγ2-containing receptors with low affinity [10,11].

Role of α1, α2 and α3-containing GABA-A receptors

The use of molecular biological techniques revealed that a single amino acid was responsible for this profound difference in affinity. The presence of a histidine residue at position 101 of the primary amino acid sequence of the α subunit (numbering for the α1 subunit, but the equivalent amino acid exists for α2, α3 and α5 subunits) conferred high affinity binding of diazepam, the presence of an arginine (in α4 and α6 subunits) conferred low affinity [12]. The amino acids were interchangeable between the subunits, conferring the appropriate affinity for diazepam without affecting any other property of the receptor. If it were possible to recapitulate this observation in an animal it would open up the possibility of making a mouse with a diazepam insensitive receptor – making diazepam more subtype selective without needing medicinal chemistry!

Using the transgenic technology of homologous recombination in mouse embryonic stem cells [13], it is indeed possible to generate such a mutated mouse, where a single targeted amino acid change has been introduced into the gene. Two groups have now taken this approach, in the first instance generating genetically modified mice where amino acid residue 101 of the GABA-A receptor α1 subunit has been changed from a histidine to an arginine [14,15]. These mice had α1-containing GABA-A receptors that were insensitive to diazepam but were otherwise perfectly normal, which is the beauty of this approach. Because this single amino acid substitution is such a subtle change to the receptor, compared to the drastic change introduced in a 'knockout' mouse – where the gene itself is functionally deleted – concerns over developmental compensatory effects that can confound interpretations of the analysis of knockout mice are avoided. These α1 histidine–arginine mice could be examined in a variety of behavioural assays to answer the question, 'which of the effects of diazepam are mediated by α1-containing GABA-A receptors?'

The group of Möhler and colleagues has generated the equivalent α2 and α3 genetically modified mice to ask the analogous questions 'which of the effects of diazepam are mediated by α2 and α3-containing receptors?' [16]. The answers to these questions have been extremely interesting. The sedative effect of diazepam [14,15], and indeed the sedative effect of the GABA-A receptor α1-selective compound, zolpidem [17] (marketed as a hypnotic by Sanofi-Synthelabo, http://www.sanofi-synthelabo.com), is ameliorated in the α1 knock-in mouse, demonstrating that sedation is mediated primarily by α1-containing GABA-A receptors. By contrast, the anxiolytic, myorelaxant and ethanol interaction effects of diazepam are retained, indicating that they must be mediated through other (i.e. α2, α3 or α5-containing) GABA-A receptors. In the α2 histidine–arginine knock-in mouse (i.e. where α2-containing GABA-A receptors are insensitive to diazepam) the anxiolytic effects of diazepam were lost [16]. This was as measured by two so-called 'unconditioned' tests of fear; (1) the light–dark box, which measures the time mice spend in a lit part of a box compared to the less threatening dark part of the box, and (2) the elevated plus maze, which measures the time spent by the mouse on the open arms of an elevated cross shaped walkway, compared to the less threatening closed arms of the cross. This does suggest that unconditioned anxiety is mediated primarily through α2-containing receptors. These studies also suggested that α2-containing GABA-A receptors mediate the myorelaxation effects of diazepam. Curiously, the role of α3-containing receptors remains unclear; removal of its diazepam sensitivity in the α3 histidine–arginine mouse did not have any effect on sedation, myorelaxation or anxiety [16].

Insights from these transgenic approaches are supported by studies with a novel subtype selective benzodiazepine site compound (L838,417) that has no activity at α1-containing receptors [15]. This compound, which positively modulates α2, α3 and α5-containing receptors, is a robust non-sedating anxiolytic, confirming that the sedative effects of benzodiazepines, but not the anxiolytic effects, are mediated primarily through α1-containing GABA-A receptors.

Cumulatively, these studies suggest that some of the effects of diazepam are indeed mediated mainly through a single receptor subtype. How can we explain such an apparently serendipitous result? Presumably, the differential distribution of the receptor subtypes in the brain is crucial. We know that the various receptor subtypes are located in different regions of the brain [18], in different types of neurons [19] and at different subcellular localizations on those cells [20], and these parameters undoubtedly determine the contribution of each receptor subtype to higher level functions.

Although the target validation studies described previously are crucial steps in the drug discovery process, clearly there is some way to go and many unanswered questions. For instance, the receptor subtypes that mediate tolerance and dependence remain unknown, and indeed these phenomena might not be restricted to any particular subtype. Although α2-containing receptors might be important in the anxiety response of mice, at least as defined by two unconditioned rodent tests, how this translates to people suffering with various anxiety disorders (e.g. generalized anxiety disorder, panic attacks, various phobias) is unknown. We need to be wary of over-extrapolating from animal behavioural models to man. Indeed it is becoming clear that for drug discovery in the area of psychiatry, a proof-of-concept bridging step is required between the preclinical models and the clinic. Along those lines, several experimental medicine models of anxiety are being developed [21–23], which could prove useful in predicting the efficacy of compounds before progressing to larger Phase II clinical trials on patient populations. Last, but not least, the huge challenge of actually producing a novel generation of GABA-A receptor subtype selective small-molecule modulators is not to be underestimated.

Role of α5-containing GABA-A receptors

The previous discussion makes no mention of α5-containing GABA-A receptors. These receptors are particularly interesting because they have quite a restricted distribution of expression, being limited primarily to the hippocampus [24,25], which is known to have an important role in learning and memory [26]. Two transgenic models with modified α5 subunits have been generated, one in which the entire subunit has been deleted [27] (α5 knockout mouse) so that there is no expression, and one in which the histidine–arginine point mutation has been introduced [28]. Inadvertently and unexpectedly, this point mutation actually led to a significant decrease in the expression of the α5 subunit in the hippocampus. Both of these genetically modified mice showed an improved performance in animal models of learning and memory, suggesting that a selective inhibitor of α5-containing receptors could have use as a cognitive enhancer, for instance in mild cognitive impaired elderly, or Alzheimer's disease patients.

Benzodiazepines such as diazepam are known to be amnesic [29]. As discussed previously, diazepam is a positive allosteric modulator, enhancing the effect of GABA. By contrast, certain experimental compounds are known (e.g. DMCM, dimethoxy-4-ethyl-β-carboline-3-methoxylate) that allosterically inhibit the effect of GABA – these are known as inverse agonists [30] and are known to enhance learning and memory in animal models [31].

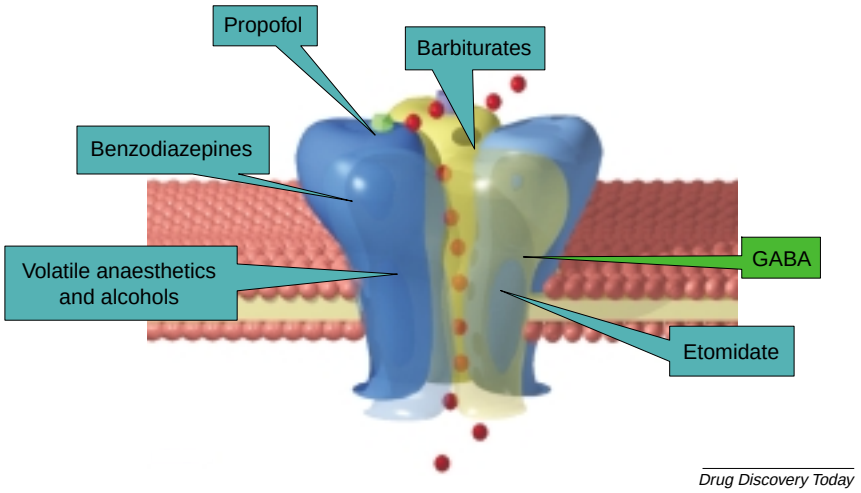

Figure 2. Cartoon representation of the γ-aminobutyric acid (GABA)-A receptor sitting in a cell membrane, indicating some of the drug binding sites. The red spots represent chloride ions flowing through the channel.

Unfortunately, like diazepam, DMCM does not show any selectivity between GABA-A receptor subtypes, inhibiting them all. As a result, it causes seizures as a result of over excitement of large parts of the brain. By contrast, the two mouse models with modified α5-subunit expression do not show any increase in seizure activity and, thus, one could predict that an α5 receptor-subtype-selective inverse agonist would also be benign.

Can we improve upon the anaesthetics?

Until now the discussion has focussed upon the benzodiazepines and how they mediate their clinical and behavioural effects through the GABA-A receptor. There are, however, numerous other pharmacological agents that act through this receptor (Fig. 2). In fact, the receptor is a pharmacologists' heaven and the potential to the pharmaceutical industry is significant. One of the important groups of therapeutic agents that act through the GABA-A receptor is the general anaesthetics, all of which act, analogous to diazepam, as positive allosteric modulators (for a review see [32]). There are two distinct types, the volatile anaesthetics such as halothane, and the intravenous (iv) anaesthetics such as etomidate and propofol. Volatile anaesthetics are primarily used for maintenance of anaesthesia, while the iv anaesthetics are primarily used for induction of anaesthesia, although they can also be used for maintenance. These are all effective reagents, but none are perfect, having their own idiosyncrasies (http://www.virtual-anaesthesia-textbook.com/). The 'hangover' effect of some of these agents can be particularly debilitating in terms of recovery from anaesthesia.

The volatile anaesthetics clearly act through a different site on the GABA-A receptor molecule to the iv anaesthetics, although the nature and location of that site remains unclear. Volatile anaesthetics, and the iv anaesthetic propofol, show no significant selectivity between any receptor subtype [32]. By contrast, the iv anaesthetic etomidate shows subtype selectivity, preferentially acting through receptors containing β2 or β3 (as opposed to β1) subunits [33]. This selectivity is determined by a single amino acid (an asparagine at amino acid number 265 in β2 and β3 subunits; a serine at the equivalent position in β1) [33]. Obvious questions arise from these findings: is the anaesthesia mediated through β2 or β3-containing receptors (or both) and would a more selective anaesthetic (i.e. acting through only β2 or β3-containing receptors) offer any clinical advantage?

There is an obvious parallel here to the previous discussion on the benzodiazepines. If one could make a genetically modified mouse, where the asparagine 265 of the β2 subunit was changed to a serine, then in that animal etomidate would act only through GABA-A receptors containing a β3 subunit. Similarly, in a genetically modified mouse where asparagine 265 of the β3 subunit is mutated, etomidate would act only through GABA-A receptors containing a β2 subunit. The question of 'which receptor subtype mediates anaesthesia?' could then be answered. The experiment using the β3-subunit-mutated mouse has now been done [34] and, remarkably, it does appear that the anaesthetic properties of etomidate are mainly mediated through β3-containing GABA-A receptors. Furthermore, it appears that the sedative properties are mediated through β2-containing receptors. The anaesthetic effect of etomidate (as measured by a paw withdrawal assay) is lost in the β3-subunit-mutated mice, and the 'righting reflex' recovered much more quickly. Extrapolating these observations to the human condition, it could be predicted that an anaesthetic that is selective for β3-containing GABA-A receptors would enable faster recovery, without perhaps the 'hangover' feeling. Given this possibility, one can see immediate advantages to both the patient and the medical community, particularly in the potential economic savings.

Of course, this all sounds good, but the realities of drug discovery and the significant challenges of developing a

receptor subtype selective small-molecule allosteric modulator, with all the physicochemical and pharmacodynamic properties required for a fast acting iv anaesthetic, are not to be underestimated. Nevertheless, clever approaches to drug target validation have again opened up new opportunities.

What about the other GABA-A receptors?

So far, we have discussed GABA-A receptors containing α, β and γ subunits. What about the subtypes that contain δ, ε, θ and π subunits? As can be seen from Fig. 1, these are the rarer subtypes of the GABA-A receptor family, and considerably less is known about their structure and function. Perhaps the best characterized of these are the δ-containing receptors (α4βδ and α6βδ) [35,36]. Neurotransmitter receptors are traditionally thought of as being located directly at the synapse, where the neurotransmitter is released by one neuron to rapidly activate the appropriate receptors on the next (so-called phasic neurotransmission). Evidence is now gathering to suggest that some neurotransmitter receptors are located in the membrane outside the synapse (extrasynaptic) where they respond to neurotransmitter that spills out of the synapse [37]. These receptors would influence the overall level of excitability of that neuron, the so called tonic neurotransmission. GABA-A δ-containing receptors could, in part, be extrasynaptic [38,39]. Certainly, the functional properties of high affinity for GABA (and therefore activated by the low concentrations of the neurotransmitter that would be found outside the synapse) and slow rate of desensitization of the channel are consistent with such a role [40,41]. So what does this mean in terms of drug targets? This is difficult to predict, particularly because we do not traditionally think in terms of modulating extrasynaptic receptors. However, it is valid to speculate that epilepsy, a complex spectrum of disorders characterized simplistically by bouts of overactivity of certain neural circuits leading to seizures [3], could be a potential application. The phenotype of the GABA-A receptor δ subunit knockout mouse, which has spontaneous seizures and shows greater sensitivity to pharmacologically induced seizures [42], would support this suggestion. Selective modulators of GABA-A δ-containing receptors (benzodiazepines do not bind to δ-containing receptors) will be crucial tools to address their potential use as drug targets.

Conclusion

The GABA-A receptor is already a validated drug target. Thus, the approaches discussed in this review represent refinement and improvement rather than *de novo* exploration of a completely novel target. Nevertheless, the paradigm described here, using sophisticated mouse genetic models in conjunction with behavioural pharmacology, has been shown to be an extremely powerful approach to define and delineate the function, and thereby the potential therapeutic use, of putative drug targets. It is likely that this approach will have wider application to exploring and validating potential drug targets in other gene families both within and outside the CNS.

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