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Review

The value of genetic and pharmacological approaches to understanding the complexities of $GABA_A$ receptor subtype functions: The anxiolytic effects of benzodiazepines

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ABSTRACT

The identification of γ-aminobutyric acid A ($GABA_A$) receptor subunit genes over the last twenty years has shown that $GABA_A$ receptors are made up of many different subtypes. As such the dissection of which receptor subtypes mediate which functions of clinically useful GABAergic drugs, such as benzodiazepines, has been extremely complicated. Two complimentary approaches have been taken: the development of subtype-selective drugs and the genetic manipulation of different receptor subunits. Both have yielded exciting results, but sometimes with contradictory findings. This review highlights the strengths and weaknesses of both approaches, illustrating with specific discussion of the work, to uncover which receptor subtype(s) mediates the anxiolytic effects of benzodiazepines.

Contents

1. Introduction

During the twentieth century great strides were made in understanding the various neurotransmitter systems that mediate all the different functional aspects of humans and animals and their modulation by drugs (Krnjevic, 2004; Stjarne, 1999). In the first half of the century this was largely determined empirically as new drugs were synthesised, tested, and their effects compared and contrasted. Many clinically useful drugs were developed despite only a basic understanding of how these compounds actually mediated their effects. As our knowledge increased over the last fifty years we began to identify the different transmitter systems and the various types of receptors through which they mediated their effects (Florey, 1991; Ortells and Lunt, 1995). The advent of molecular biology held the promise of definitively identifying all the different receptor subtypes and combined with advances in pharmacology, cell biology and biochemistry meant that we should soon understand exactly how these systems worked. For many neurotransmitters the biological truth of the situation turned out to be far more complicated than expected, none more so than for the γ-aminobutyric acid (GABA)-ergic system (Whiting et al., 1995).

$GABA_A$ receptors are the target of multiple classes of clinically useful drugs (benzodiazepines, barbiturates, general anaesthetics) that produce anxiolysis, sedation, anaesthesia, amnesia, muscle relaxation and seizure protection. These very widely used drugs were largely discovered and developed before we understood the complexity of the $GABA_A$ receptor (Nemeroff, 2003). This is perhaps the chief reason why they share a number of undesirable effects such as tolerance and dependence and, depending upon the situation,

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cognitive impairment, sedation and ataxia. Our understanding of the $GABA_A$ receptor has increased enormously over the last three decades, but is still far from complete. In terms of general clinical practice, this improved understanding has so far only yielded more selective sedative drugs, such as zolpidem (Sanger, 2004).

The true complexity of the $GABA_A$ receptor was first appreciated towards the end of the 1980s as molecular biologists started to clone the different subunit cDNAs of the different receptor genes (Levitan et al., 1988; Schofield et al., 1987). It emerged that there were six different α subunits, 3 β, 3 γ and several other subunits which were generally less abundantly expressed: δ, π, θ and ε. The $GABA_A$ receptor is a ligand-gated ion channel made up of five subunits with a central chloride-permeable pore. The majority of receptors are made up of two α, two β and one γ subunits. The expression patterns of these different subunits were first determined using *in situ* hybridisation (Laurie et al., 1992; Persohn et al., 1992; Wisden et al., 1992) and later confirmed via immunohistochemistry (Pirker et al., 2000; Schwarzer et al., 2001). In addition, immunoprecipitation studies indicated the subunit composition and abundance of the more highly expressed receptor subunit combinations (Whiting et al., 1995). For example, approximately 50% of all the $GABA_A$ receptors are made up of α1β2γ2 subunit combinations (McKernan and Whiting, 1996). Whilst all these studies are important for understanding the basics of $GABA_A$ receptor pharmacology they do not give any indication as to the function of the different subtypes in a whole animal.

The plethora of available drugs that modulate $GABA_A$ receptors were all found to modulate more than one receptor subtype. Most of these drugs are allosteric modulators, although some classes such as barbiturates and anaesthetics also directly activate the receptor at higher concentrations (Franks and Lieb, 1994; Jackson et al., 1982). The pharmacological profiles of the separate drug classes do differ, but these differences are insufficient to clearly associate a given functional effect with a specific receptor subtype. For example, clinically used benzodiazepine-site ligands (e.g. diazepam, chlordiazepoxide) only potentiated receptors containing α1, α2, α3 or α5 in combination with a γ2 subunit. They have no effect on α4- or α6 containing receptors (with or without an accompanying γ2 subunit), nor do they activate γ1- or δ-containing receptors (for review see Whiting et al., 1995). Although benzodiazepine-site ligands only activate four receptor subtypes, these make up ~75% of all $GABA_A$ receptors in the brain and are expressed in most brain regions (McKernan and Whiting, 1996), thus it is not clear which subtypes mediate which functions. Benzodiazepine-site ligands can be split into two distinct classes: the first is those that have a benzodiazepine chemical structure at their core (e.g. diazepam), and the second that have a non-benzodiazepine structure (e.g. zolpidem, L-838,417). Of greatest importance for this review is that both classes bind to the benzodiazepine site on the $GABA_A$ receptor and allosterically modulate the response of the channel to GABA (Atack, 2005). Therefore, use of the term benzodiazepines (BZs) here refers to both classes, regardless of chemical structure.

In the last 15 years two approaches have been taken to reveal the function of the different receptor subtypes: the development of genetically modified mice and the synthesis of subtype-selective compounds. Both methods have their merits, but neither alone has been able to definitively show which receptor subtypes mediate which effects, indeed they have sometimes produced conflicting answers.

2. Advantages and disadvantages of pharmaceutical and genetic approaches

The pharmaceutical approach has the enormous practical advantage that research findings in animals should enable development of marketed drugs which offer superiority over the existing agents and therefore benefit to the patient. Novel compounds can, of course, be administered to a range of species, thus giving flexibility to choose the most suitable species for the particular function being investigated, for example primates are better suited to complex cognitive tasks than rodents. Finally the administration of compounds can be tightly controlled temporally, thus facilitating studies where a baseline in the absence of drug can be recorded, then the effect of the administered drug observed and if desired, tracked until the response returns back to baseline as the drug is cleared from the body. The pharmaceutical approach does however have one major limitation in that all of the above are only possible if you actually manage to make a compound that has the desired selectively. For $GABA_A$ receptors this has proved a significant challenge because of the high degree of similarity between the different subunits (Wingrove et al., 2002).

One of the great strengths of the genetic approach is that each of the 16 subunits is encoded by a separate gene (Barnard et al., 1998) and therefore amenable to genetic manipulation individually, hence the selectivity issues seen with drugs are not necessarily a problem. Knockout (KO) mice, where a given gene is disrupted resulting in the absence of expression of that particular subunit, have been successfully generated for many of the α subunits, β2, β3, γ2 and δ. Some of these have suffered from embryonic/neonatal lethality (β3, γ2; (Culiat et al., 1995; Gunther et al., 1995; Homanics et al., 1997), which gives a clear indication of their importance during development, but does not define their function in an adult animal. There are also problems with compensation by other $GABA_A$ subunits e.g. increased expression of α2 and α3 subunits in α1 KO mice (Korpi et al., 2002; Kralic et al., 2002a; Peng et al., 2002; Sur et al., 2001). Additionally, unrelated ion channels may have altered expression to compensate for the loss e.g. increased potassium leak current via TASK-1 channels in α6 KO mice (Brickley et al., 2001). Additionally, alterations in the expression levels of flanking genes have been observed in α6 KO mice (Uusi-Oukari et al., 2000). A more elegant approach is making a single point mutation in a given subunit that will not affect the normal function of the subunit, but will disrupt pharmacological modulation of it. This approach has been very powerful for determining the effects of BZs at different BZ-sensitive receptor subtypes (for review see Mohler et al., 2002) and also understanding the actions of the general anaesthetics etomidate and propofol which are β2/β3-selective (Jurd et al., 2003; Reynolds et al., 2003b). Another advantage of mutant mouse lines is that they can be interbred to produce double or even triple mutants, although this is not a trivial undertaking because of the practicalities of the breeding strategy. Double/triple mutants offer the possibility of creating specific combinations of effects that could not be achieved with a drug. The selectivity profile of a drug is determined by the shape of the binding pocket and/or efficacy transduction mechanism of the receptor, thus there are limitations in the combinations of selectivity that can be achieved pharmaceutically. One final limitation of the genetic approach is the largely permanent nature of the mutation. Advances in the technology does now allow genes to be switched on/off using inducible systems such as TetOn/TetOff (Sprengel and Hasan, 2007), but the time course and specificity of these is no match for exogenously administered drugs.

The above two paragraphs describe the pros and cons of the pharmaceutical and genetic approaches. Great advances have been made in understanding the functions of the different $GABA_A$ receptor subtypes by combining these two powerful approaches. Understanding how the anxiolytic effects of BZs are mediated is probably one of the more important findings, as this is an area that would really benefit from improved clinical drug treatments.

3. The anxiolytic effects of BZs

Early studies comparing the actions of the α1-preferring BZ-like drug zolpidem with non-selective BZs had indicated that zolpidem was a much more potent hypnotic than anxiolytic (Depoortere et al., 1986). Thus it appeared unlikely that α1-containing receptors

mediated the anxiolytic properties of BZs. However, zolpidem is also a full agonist BZ at α2- and α3-containing receptors, although it has a lower affinity at these particular subunits (K_i α1 = 23 nM, α2 = 110 nM, α3 = 320 nM, α5 > 10 μM; Smith et al., 2001). Thus more selective tools are required to definitively determine which subtype mediated the anxiolytic effects of BZs. Although many pharmaceutical companies had tried to produce affinity-selective compounds for the four different BZ-sensitive receptors (α1βγ2, α2βγ2, α3βγ2 and α5βγ2), this approach has met with limited success. A careful look at the amino acid sequences of the different α subunits combined with knowledge of the binding site of BZs indicates why this was the case. The BZ binding pocket lies at the interface of the α and γ2 subunits. Both subunits contribute key amino acids for BZ binding, for example phenylalanine at position 77 and methionine-130 on the γ2 subunit (Sigel, 2002). On the α subunit histidine-101, tyrosine-159 and -209 and threonine-206 are particularly important and modification of these significantly reduces BZ binding affinity (Amin et al., 1997; Buhr et al., 1997; Wieland et al., 1992). These four critical amino acids for BZ binding are common to all the BZ-sensitive α subunits and likewise the γ2 subunit is required for BZ sensitivity. The highly conserved BZ binding pocket indicates why it has been very difficult to design novel compounds that bind with significantly higher affinity to α1, α2 or α3 subtypes compared with the others. The exception to this is α5, where binding selectivity has been achieved (Liu et al., 1996; Quirk et al., 1996; Skolnick et al., 1997). However, a potentially significant breakthrough in developing a binding-selective compound was recently published by Selleri and co-workers (called Compound 4; Selleri et al., 2005). They found that relatively small modifications to the structure of zolpidem yielded a compound with high α1 affinity (K_i = 31 nM) and > 10 μM affinity at α2-, α3- and α5-containing receptors (Selleri et al., 2005). Interestingly this extremely α1-selective compound was anxiolytic in mouse light/dark box and rat Vogel conflict tests, which is in contrast to many other studies (see below). Further studies from other laboratories using this compound have not yet been published.

With the above information in mind researchers took two different approaches to determine the functional role of the different $GABA_A$ receptor subtypes: genetic manipulation of the different subunits or synthesis of novel subtype-selective compounds.

4. The genetic approach

The first GABA mutant mice generated were the KO mice, where expression of one of the $GABA_A$ receptors had been ablated by disruption of the gene sequence. Knocking out either γ2 (Gunther et al., 1995) or β3 (Culiat et al., 1995; Homanics et al., 1997) subunits produced mutant mice with severe deficits and many of them died as neonates. In contrast, disruption of α1 (Kralic et al., 2002a,b; Sur et al., 2001), β2 (Sur et al., 2001) or α6 (Jones et al., 1997) did not produce an obvious phenotype in the homozygous KO mice despite these subunits being widely expressed (α6 is restricted to the cerebellum; (Pirker et al., 2000). Whilst being interesting models to study the role of $GABA_A$ receptor subtypes during development, these particular models only made a small contribution to understanding the subtypes involved in anxiolysis. The γ2 heterozygous KO mice displayed neophobia in a novel environment (Crestani et al., 1999), which confirmed this subunit's role in anxiety-related behaviours. However, given that all BZ-sensitive receptor subtypes contain a γ2 subunit this finding was perhaps not unexpected. The analysis of the effects of non-selective BZs, such as diazepam or chlordiazepoxide, in the α1 mutant mice should have helped determine whether activity at α1 receptors was required for anxiolysis. The results of these studies were highly surprising: diazepam was far more sedating in α1 KO mice compared with wildtype (WT) controls (Kralic et al., 2002a,b; Reynolds et al., 2003a). This result was found by two separate laboratories using two independently generated α1 KO mouse lines. Other evidence (McKernan et al., 2000; Rudolph et al., 1999) had suggested that α1 mediated the majority of the sedative effects of BZs, yet the KO studies indicated the opposite. Although the precise reason for this discrepancy has not been identified it is likely due to compensation by other GABA subunits (Kralic et al., 2002a,b; Sur et al., 2001) or possibly other unrelated ion channel expression changes as was seen in the α6 KO mouse (Brickley et al., 2001). The generation of α5 KO mice demonstrated that α5-containing receptors were not required for anxiolysis, but were involved in cognition (Collinson et al., 2002).

A more sophisticated genetic approach was the generation of point mutation, or knock-in (KI), mice. In these studies the histidine at position 101 was mutated to an arginine. His-101 is critical for BZ binding and is replaced by Arg in the BZ-insensitive subunits α4 and α6 (Wieland et al., 1992). Thus by exchanging His-101 (or its equivalent position in α2, α3 or α5) for Arg, KI mice can be generated with altered sensitivity to BZs in the mutated subunit. In theory there should be no compensatory changes in these mice, because the function of the receptor and its response to GABA remains unaltered — only its sensitivity to BZs should change. In practice some minor changes in receptor expression or differences in response of WT and mutant mice have been observed, but these are probably much reduced compared with KO mice (Crestani et al., 2002; Morris et al., 2006). α1H101R (McKernan et al., 2000; Rudolph et al., 1999), α2H101R (Low et al., 2000; Wafford et al., 2004), α3H126R (Low et al., 2000) and α5H105R (Crestani et al., 2002) have all been generated and studied to determine which subtype mediates the anxiolytic properties of BZs. Studies with independent α1H101R mouse lines agreed that α1-containing receptors mediated sedation, amongst other things, but not anxiolysis (McKernan et al., 2000; Rudolph et al., 1999). Likewise the α5-containing receptors did not appear to be involved in the anxiolytic effects of BZs using the plusmaze and light/dark box tests of unconditioned fear (Crestani et al., 2002). Studies using α2H101R and α3H126R mice in these anxiety tests suggested that α2 mediated anxiolytic effects of BZs, whereas α3 did not (Low et al., 2000). However, some debate arose as to whether changes in locomotor activity caused by diazepam in these tests confounded this interpretation (Crestani et al., 2001; Reynolds et al., 2001). Certainly the plusmaze and light/dark box tests rely on the locomotor activity of the mouse exploring the novel test apparatus to provide information about its anxiety state and this is susceptible to producing false positive or negative results (Dawson et al., 1994; File, 1990). However, it was not clear whether or not this was actually the case in the experiments using α2 and α3 KI mice.

In order to clarify the role of α2 and/or α3 in anxiety, additional approaches were necessary beyond the use of non-selective BZs with point mutations in single α subunits. Triple KI mice have been bred, but none of the studies reported to date (Fradley et al., 2007; Prenosil et al., 2006) have investigated anxiolytic effects.

5. The pharmaceutical approach

As noted above, apart from Compound 4 (Selleri et al., 2005) only limited selectivity has been obtained in terms of affinity of binding between α1, α2 and α3. There are two other ways in which selectivity could be achieved: partial agonism and selective efficacy. Partial agonism is a well described phenomenon for both direct agonists and also for allosteric agonists (Bowery, 2006). For example, bretazenil was developed as a partial agonist BZ that did not differentiate between any of the BZ-sensitive receptor subtypes (α1-, α2-, α3- and α5-containing receptors), but only possessed ~20–44% efficacy in electrophysiological tests compared with clinically used BZs (Smith et al., 2001). In this way it was hoped to achieve anxiolysis without the accompanying side effects of standard BZs. Although this differentiation was achieved in animal studies (Haefely et al., 1990) it did not translate through to humans (van Steveninck et al., 1996). Efficacy selective BZs generally bind to all the BZ-sensitive receptor subtypes

with roughly equivalent affinity i.e. they are *not* selective in terms of binding affinity. Instead, selectivity is achieved because they only produce an allosteric agonist/inverse agonist effect in a subset of those receptors to which they bind. At the other receptor subtypes there is no net agonist or inverse agonist effect (i.e. they are antagonists) and thus that compound is functionally silent at that particular subtype (Atack, 2005). Precisely which amino acids are important for the transduction of BZ binding to produce an effect on the open probability of the $GABA_A$ channel is not fully understood, but empirical studies have indicated that this is possible.

Over the last few years several efficacy-selective compounds have been published (Atack et al., 2005, 2006a,b; Dias et al., 2005; Griebel et al., 2001; McCabe et al., 2004; McKernan et al., 2000; Rabe et al., 2007; Rowlett et al., 2005) with differing efficacy profiles. Some of these compounds, such as pagoclone (Atack et al., 2006a) and SL651498 (Griebel et al., 2001), show differing levels of agonist efficacy between the different subtypes, but still have some level of efficacy at all of the four BZ-sensitive receptor subtypes. For example, SL651498 is a full agonist at α2- and α3-containing receptors with lower efficacy (~45% that of zolpidem) at α1- and α5-containing receptors (Griebel et al., 2001). Other compounds display partial agonism at some receptors (e.g. α2 and α3 for TPA023) and are antagonists at others (α1 and α5 for TPA023). Data generated using these efficacy-selective compounds demonstrated that α1 activation was not necessary to produce an anxiolytic effect in a range of animal models. In rats unconditioned fear models such as plusmaze have shown positive anxiolytic-like effects of α2-, α3-, α5-selective (L-838,417; McKernan et al., 2000), α2-, α3-selective (TPA023; Atack et al., 2006b) and α3-selective (TP003, Dias et al., 2005; ELB139, Langen et al., 2005) compounds. Complimentary studies using an α3-selective inverse agonist (α3IA; Atack et al., 2005) indicated that this compound was anxiogenic on the plusmaze. Together these studies suggested that compounds with agonistic efficacy at α3-containing receptors were sufficient to produce anxiolysis. Additional studies with ELB139, TP003, TPA023 and L-838,417 indicated that anxiolytic effects were also seen in the rat conditioned fear tests such as Vogel conflict (Langen et al., 2005), fear-potentiated startle (Atack et al., 2006b; McKernan et al., 2000), conditioned suppression of drinking (McKernan et al., 2000) and conditioned emotional response (Mathiasen et al., 2007). Furthermore, anxiolytic effects were also observed in mice (Dias et al., 2005; Mathiasen and Mirza, 2005), squirrel monkeys (Atack et al., 2006b; Dias et al., 2005) and rhesus monkeys (Rowlett et al., 2005). As a body of work these preclinical studies all suggested that a compound with α3 agonism would be effective in treating generalised anxiety disorder in the clinic.

Unfortunately, none of the subtype-selective compounds described above have absolute selectivity of α3 over α2. TP003 possesses a high degree of selectivity, with relative agonist activity of 83% at α3 and <15% at α2 (for details see Dias et al., 2005). The <15% potentiation at α2 was not able to produce an anxiolytic effect in the stress-induced hyperthermia test in α2H101R KI mice (Dias et al., 2005), but it is possible that the low level of α2 efficacy augmented the anxiolytic effect mediated through α3 in WT animals. Generation of new exquisitely selective compounds or further detailed studies using a range of tests are required to fully pick apart this possibility.

An additional complication is that anxiety-like behaviours are not unitary. At the simplest level they can be split into conditioned (learnt) and unconditioned (innate) anxiety responses and these are mediated by different brain regions (LeDoux, 2000; Toufexis, 2007). The amygdala is important for conditioned anxiety, whereas the bed nucleus of the stria terminalis is more involved in unconditioned anxiety (Walker et al., 2003). These two brain regions express a different compliment of $GABA_A$ receptor subunits (Pirker et al., 2000) and therefore different receptor subtypes may be responsible for mediating different kinds of anxiety response. A full discussion of the different types of anxiety responses is beyond the scope of this review and has been discussed elsewhere (LeDoux, 2000; Walker et al., 2003).

6. The combined approach

A recent elegant study by Morris and co-workers showed that in WT mice diazepam was anxiolytic in a conditioned emotional response test, but that this effect was lost α2H101R KI mice (Morris et al., 2006), thus supporting the original KI data generated by Low et al. (2000). Importantly these authors went on to run the same test using a subtype-selective compound, L-838,417, which was anxiolytic in *both* the WT and α2H101R KI mice. These data in the same test and same group of trained mice indicated that α2-containing receptors were necessary for anxiolytic effects when using diazepam, but were not required when using an α2-, α3- and α5-selective compound. These data raise an important consideration when interpreting data from genetic and pharmacological sources in determining which receptor subtypes mediate a given effect of BZs. The genetic studies mostly used the subtraction method of utilising a non-selective compound (usually diazepam) and removing some of its activities via genetic manipulation (H→R mutations). If a given functional effect was absent then that particular subtype was interpreted to mediate the behaviour under study. However, the behaviour observed in a drug-treated animal is the sum of all the different effects of that drug. Thus a BZ produces (to varying degrees dependent upon dose, selectivity and functional efficacy) sedation, ataxia, muscle relaxation, anxiolysis, impairments in memory consolidation and increased pain and seizure thresholds. Many of these are contributing factors to the overall behavioural response in anxiolytic tests in animals. Certainly drug/transgenic studies have indicated that BZ-mediated sedation is increased in the absence of α2 activity (Morris et al., 2006; Wafford et al., 2004). The hypothesis put forward by Morris and colleagues to explain the apparently contradictory results in their study was that higher receptor occupancy of α3-containing receptors may be required to produce anxiolysis compared with α2-containing receptors. Where the non-selective compound diazepam was used in the α2 KI mice the α3-mediated anxiolytic effects were masked by α1-mediated sedation generated at lower receptor occupancy and thus were not observed in the test (Morris et al., 2006). However, the subtype-selective compound did not possess α1 activity and therefore was able to produce anxiolytic effects through α3-containing receptors without interference from α1-mediated sedation. Careful testing of this hypothesis will be required in future studies in order to resolve this apparently conflicting result.

In summary, both genetic and pharmacological approaches are very valuable for probing into the complexities of $GABA_A$ receptor-mediated behaviours. The combined power of these approaches allows more detailed interpretation of the results and hopefully in the future there will be more studies that take the combined approach in order to work out exactly which subtypes mediate which functions.

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