Differentiating the role of \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor subtypes

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Abstract
The inhibitory tone maintained throughout the central nervous system relies predominantly on the activity of neuronal GABA\(_A\) (\(\gamma\)-aminobutyric acid type A) receptors. This receptor family comprises various subtypes that have unique regional distributions, but little is known about the role played by each subtype. The majority of the receptors contain a \(\gamma_2\) subunit and are sensitive to modulation by BZs (benzodiazepines), but differ with regard to \(\alpha\) and \(\beta\) subunits. Mutagenesis studies combined with molecular modelling have enabled a greater understanding of receptor structure and dynamics. This can now be extended to \textit{in vivo} activity through translation to genetically modified mice containing these mutations. Ideally, the mutation should leave normal receptor function intact, and this is the case with mutations affecting the BZ-binding site of the GABA\(_A\) receptor. We have generated mutations, which affect the BZ site of different \(\alpha\) subunits, to enable discrimination of the various behavioural consequences of BZ drug action. This has aided our understanding of the roles played by individual GABA\(_A\) receptor subtypes in particular behaviours. We have also used this technique to explore the role of different \(\beta\) subunits in conferring the anaesthetic activity of etomidate. This technique together with the development of subtype-selective compounds facilitates our understanding of the roles played by each receptor subtype.

Introduction
GABA\(_A\) (\(\gamma\)-aminobutyric acid type A) receptors are a major member of the ligand-gated ion-channel family and are the primary means for exerting inhibitory control throughout the central and peripheral nervous systems. A great deal is now known about the molecular composition of these receptors and how the distinct subtypes are constructed from a family of 18 different subunits to form the characteristic pentameric channel–protein complex with a central chloride-selective pore [1–3]. Expression studies of different subunit combinations have clearly defined some of the pharmacological differences between specific receptor combinations that are thought to be expressed \textit{in vivo}, and now we have a clearer understanding of at least some of those amino acid residues, which contribute to ligand-binding domains and channel function at the receptor level [4]. It is possible to utilize these pharmacological and physiological discoveries in combination with crystallographic data and molecular modelling to hypothesize the protein structure and physical domains for ligand interactions [5,6]. We can also combine these findings together with recombinant genetics to make single mutations or manipulations in targeted subunit proteins in mice, to study the \textit{in vivo} consequences of alterations in specific subunits [7]. In the present study, we have used this technique to investigate the role played by individual GABA\(_A\) receptor subtypes in the behavioural actions of BZs (benzodiazepines) and anaesthetics.

BZ regulation of GABA\(_A\) receptor subtypes
A majority of GABA\(_A\) receptors are sensitive to modulation by BZs. Presence of a \(\gamma\) subunit is essential and most of the receptors contain a \(\gamma_2\) subunit that confers the highest degree of sensitivity. The less abundant \(\gamma_1\) and \(\gamma_3\) subunits confer decreased sensitivity, but do show a degree of modulation by a subset of BZ-site ligands [8,9].

It is now believed that each pentameric receptor contains a single copy of the \(\gamma_2\) subunit and that the BZ-binding site is formed in a pocket between this and an \(\alpha\) subunit [6]. Interestingly, a number of mutagenesis studies have revealed that amino acid residues on the \(\gamma_2\) subunit, which are important contributors to this pocket are in homologous positions with those on the \(\alpha\) subunit, which form the binding pocket for GABA [10]. This suggests that these binding pockets may be structurally quite similar but located between different subunits.

To date, several residues have been identified, which form part of the binding site [6]. In the \(\gamma_2\) subunit, a key residue is Phe-77, which contributes a major component to the binding of imidazopyridines and \(\beta\)-carbolines; however, Met-130 was found to be more important for binding of flunitrazepam and diazepam [11]. The \(\alpha\) subunit also contributes a vital section of the binding pocket, particularly a histidine residue located at position 101 in the \(\alpha_1\) subunit [12]. This residue is a histidine in \(\alpha_1, \alpha_2, \alpha_3\) and \(\alpha_5\) but arginine in \(\alpha_4\) and \(\alpha_6\), which have a modified binding site less sensitive to ‘classical’ BZs such as...
diazepam, but still sensitive to other BZ ligands such as Ro15-4513 and flumazenil, where these compounds now behave as agonists [13].

The α subunit contributes other key residues to make up the binding pocket, such as tyrosine residues at positions 159 and 209 and a threonine residue at position 206 in α1. Modification of these residues significantly decreases BZ affinity [14].

The behavioural consequences of BZ agonist modulation include anxiolysis, sedation, amnesia, tolerance, possible abuse potential and interactions with ethanol. The presence of multiple subtypes within the brain, which are anatomically distinct, suggest that separate functions are probably controlled by different mechanisms. As a consequence, subtype selectivity might lead to a separation of these different behaviours. The in vivo effects of the hypnotic compounds zolpidem and other α1 selective agents suggest that α1 contributes more of a sedating component compared with non-selective BZ agonists, supporting this hypothesis for differential function [15]. Recently, we have used mutagenesis techniques combined with gene targeting to generate mice with α subunit mutations at the BZ-dependent histidine residue, enabling the differentiation of BZ-mediated behaviours according to the α subtype [16].

The α1His-101 mutant mouse
A particular advantage of using the histidine mutation is that when this residue is mutated to an arginine, normally present at this position in α4 and α6, normal functioning of the receptor is unaffected. The expression levels and anatomical distribution of the subunit are identical with WT (wild-type) and the physiological properties of the receptor are unchanged [16,17]. This ‘silent mutation’ means that there is no apparent phenotype in the mice until they are challenged with a BZ compound. Depending on the action of the compound at the mutated receptor this will determine the response of α1-containing receptors to the compound, and differences in behaviour will reflect this altered pharmacology.

To study the in vitro phenotype of α1-containing receptors in these mutant mice, we performed electrophysiological recordings from cerebellar Purkinje neurons, which have previously been shown to express predominantly α1β2γ2 GABA_A receptors. Maximum responses to GABA application were not different from that of the WT; however, modification of the submaximal EC20 by different BZ ligands was quite different (Figure 1). Unlike the WT cells, responses in the mutant mice were not modulated by diazepam or chlordiazepoxide. In contrast, Ro15-4513 and flumazenil, which behave as antagonists at WT receptors, produced large potentiation of GABA responses in the mutant mice. In addition, bretazenil became more efficacious in the mutant mice when compared with WT, which correlates with the effects of this mutation in recombinant receptors [13]. In vivo studies have shown that these mutant mice are much less sedated by diazepam when compared with WT mice in standard tests of sedation, such as the rotarod test [16]. These results support the hypothesis that BZ agonist activity at α1-containing receptors produces the sedating effects associated with these compounds.

Furthermore, diazepam actually produces a marked increase in spontaneous activity/exploration when mutant mice are introduced to a novel environment (e.g. a testing chamber) when compared with WT which showed no change [16]. Whereas this additional data further supports the hypothesis that α1 mediates the sedative effects of BZs, it does create a problem with regard to investigating the anxiolytic effects of these drugs: most anxiolytic tests for mice rely on locomotor activity. For example, the elevated plus maze and light/dark box tests evaluate the amount of time a mouse spends in the open, ‘exposed’ areas of the test apparatus compared with the enclosed, ‘safer’ areas of the apparatus. Anxiolytic drugs increase the time spent in those open areas, but marked changes in activity between the two groups can confound the interpretation of the data. Thus we were unable to confirm that the α1 mutant mice are still sensitive to the anxiolytic properties of BZs.

Other α subunit histidine mutants
Equivalent mutation of this histidine residue has been made in the other BZ-sensitive α subunits and the mutant mice generated express these receptors. Consequences of histidine mutation in α2 and α3 subunits was reported by Low et al. [18]. These authors used diazepam in the light/dark box test to investigate the anxiolytic activity of diazepam. They found...
that unlike the WT or α₁H126R mice, the α₂H101R mice did not demonstrate an anxiolytic response to diazepam. This was recapitulated in the elevated plus maze test suggesting that receptors containing α₂ but not α₁ mediate the anxiolytic effects of BZs. However, as noted above, these results may be confounded by changes in activity levels of the two groups of mice [19]. We have investigated the role the α₂ subunit plays in novelty-induced exploration and found that α₂ mutant mice are actually more suppressed by diazepam when compared with WT controls (Figure 2). These results suggest that the α₂ subtype mediates the stimulatory properties of BZs, which are normally counterbalanced by the sedative effects mediated through α₁. Thus, in the α₁ mutant mouse, only the stimulatory effects are present and increased activity is seen, whereas in the α₂ mutant mice only the sedative component is present and so a greater level of sedation is observed.

The α₃ subunit has also been subject to mutation at this position and found not to contribute to the sedative, anxiolytic or anticonvulsant effects of diazepam. This receptor is expressed for the most part extrasynaptically, and anxiolytic or anticonvulsant effects of diazepam. This is most abundant in the hippocampus where it has recently been shown to mediate a tonic conductance in hippocampal pyramidal neurons [20]. α₃ knockout studies suggest the involvement of this subunit in cognitive processing of spatial memory [21] and contingent with this, the α₃H105R mice display a facilitation of trace fear-conditioned learning [22]. In the latter mouse model, the introduced H105R point mutation resulted in an unexpected selective reduction of α₃-containing GABAₐ receptors predominantly in the hippocampus, so that this mouse model can serve as a partial α₃ gene knockout. However, we generated α₁H105R mutant mice by a similar, but not identical targeting strategy (R. Fradley and T.W. Rosahl, unpublished work). As expected, no reduction of α₃-containing GABAₐ receptors in the hippocampus or in the whole brain were found in our mouse model as determined by quantitative radioligand binding assays using [³H]Ro15-4513 (A. Smith, J. Atack and T.W. Rosahl, unpublished work).

In general terms, this single-point mutant technology in genetically modified mice has provided a useful technique to study the contribution of individual receptor subtypes to BZ-mediated behaviours and suggests that subtype selective compounds would be most beneficial and lack some of the adverse side-effects associated with currently available non-selective compounds [23,24].

GABAₐ subunits and anaesthesia

In recent years, there has been an accumulating amount of evidence for the involvement of GABAₐ receptors in the action of anaesthetics. The majority of intravenous and volatile anaesthetics potentiate the action of GABA at inhibitory synapses at concentrations that correlate well with their in vivo potency [25]. In addition, stereoisomers with differential anaesthetic activity match their in vitro effects at GABAₐ receptors [26]. Recent identification of critical residues on GABAₐ and glycine receptor subunits for volatile anaesthetics and alcohols reveal that these compounds may well act by binding to ligand-gated ion channels [27]. The intravenous anaesthetic etomidate shows GABAₐ subtype selectivity in vitro for β₂- and β₃-containing receptors when studied on recombinant receptors [28]. This selectivity is determined through a single amino acid residue Asn-265 in the transmembrane 2 domain of the receptor [29]. Recently, we have utilized genetic manipulation to generate a mouse containing a mutation in the β₂ subunit at this position rendering β₂-containing receptors less sensitive to etomidate [30].

As with the histidine mutation, this single amino acid switch is also effectively silent with regard to normal GABAAergic function. In Purkinje neurons, the amplitude of GABA-induced currents was similar to WT but, unlike WT, responses were not modulated by etomidate. Similarly, synaptic currents were not different but the prolongation of decay induced by etomidate was significantly reduced in the β₃N265S mice. The anaesthesia following administration of etomidate was determined by measuring the sleep time (time to recover righting reflex) and was similar to that in WT mice, revealing that β₃-containing receptors contribute a major part of the anaesthetic effect of etomidate. At lower doses, which produced sedation in WT mice, no effects were observed in the β₃N265S mice. In addition, after recovering from anaesthesia the mutant mice recovered their motor abilities much more rapidly than WT and exhibited less slow
wave sleep after anaesthesia. Etomidate also produced a large hypothalamic effect in WT mice, which was reduced in the β2N2655 mice, and this effect may contribute to their rapid recovery from anaesthesia [30].

An equivalent mutation in the β3 subunit (β3N265M) has also been generated and demonstrates that indeed the righting reflex after etomidate is profoundly affected in this mouse, as well as an abolition of the anaesthetic ability to prevent the paw withdrawal reflex [31]. These results together suggest that the β3-containing receptors are the primary mediators of the anaesthetic effects of etomidate and this may well be generally applicable to other anaesthetics, whereas β2, probably in combination with α1, mediates the sedating effects of etomidate. The identification of residues, which affect the in vitro potentiation by volatile anaesthetics, suggest other mutants that might be useful in the investigation of these types of drugs.

The ability to selectively affect function of these compounds suggests that we will soon learn a great deal more about the mechanism of action of anaesthetics and pathways which mediate the various effects produced by anaesthetics in vivo. As we learn more about the structure function of ligand-gated ion channels at the molecular level, we can now apply this knowledge using transgenic technology to manipulate receptors in vivo. This provides us with useful tools to investigate the parts of the central nervous system, which mediate specific behaviours and identify the role of different ion channels in behaviour. Hopefully, this will open up a whole new arena of study in the field of ion channels and enable a much clearer understanding of how psychoactive drugs mediate their effects on the central nervous system.

References


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