GABA<sub>A</sub> receptor subtypes: dissecting their pharmacological functions

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The enhancement of GABA-mediated synaptic transmission underlies the pharmacotherapy of various neurological and psychiatric disorders. GABA<sub>A</sub> receptors are pluri- potent drug targets that display an extraordinary structural heterogeneity: they are assembled from a repertoire of at least 18 subunits (α1–6, β1–3, γ1–3, δ, ε, θ, ρ1–3). However, differentiating defined GABA<sub>A</sub> receptor subtypes on the basis of function has had to await recent progress in the genetic dissection of receptor subtypes in vivo. Evidence that the various actions of allosteric modulators of GABA<sub>A</sub> receptors, in particular the benzodiazepines, can be attributed to specific GABA<sub>A</sub> receptor subtypes will be discussed. Such discoveries could open up new avenues for drug development.

The enhancement of neuronal inhibition by GABA is one of the most powerful therapeutic strategies for the treatment of CNS diseases such as generalized anxiety disorders, sleep disturbances, muscle spasms and seizure disorders. GABA<sub>A</sub> receptors are targets for many drugs in wide clinical use; these include ligands of the benzodiazepine site of the GABA<sub>A</sub> receptor, barbiturates, anesthetics and – currently at an experimental stage – neurosteroids. GABA<sub>A</sub> receptors are ubiquitous in the CNS (Ref. 1). Therefore, a major goal in neuropharmacology has been to target drugs selectively to defined GABA<sub>A</sub> receptor subtypes and thereby refine the therapeutic spectrum of the presently available drugs, reduce their side-effects and discover new therapeutic indications.

GABA<sub>A</sub> receptors are pentameric membrane proteins that operate as GABA-gated Cl⁻ channels. These receptors are most clearly distinguished by their subunit architecture, which in mammalian brain comprises seven different classes of subunits with mostly multiple variants (α1–6, δ–ε, θ, ρ1–3). Most GABA<sub>A</sub> receptors are composed of α-, β- and γ-subunits. Two mutational analyses of multiple recombinant GABA<sub>A</sub> receptors have generated valuable information on their drug sensitivity in vitro. However, pharmacological analysis of GABA<sub>A</sub> receptor subtypes has had to wait for the generation of animal models in which particular GABA<sub>A</sub> receptor subunits are either inactivated (knockout strategy) or selectively point-mutated (knock-in strategy). The lessons for drug design learned from these approaches will be discussed in the present article.

GABA<sub>A</sub> receptors analyzed by gene-knockout strategies
In gene-knockout strategies, ablation of a particular receptor subunit would be expected to perturb the structure of a defined group of GABA<sub>A</sub> receptors and thereby facilitate the genetic dissection of receptor subtypes in vivo.
cause a corresponding alteration in the physiology and pharmacology of the mutant mice. The best studied examples of this are mice with targeted mutations of the genes encoding α6-, β3-, δ- or γ2-subunits.

Targeting the gene encoding the α6-subunit
GABA<sub>ᵦ</sub> receptors that contain the α6-subunit are expressed exclusively in a single type of neuron, the cerebellar granule cell. Studies on knockout mice that lack the α6-subunit reported no change in the response of these mice to pentobarbital, general anesthetics or ethanol, compared with wild-type mice, but the knockout mice were more sensitive to the motor-impairing action of diazepam in an accelerating rotarod test (although in a limited dose range only) than their wild-type counterparts. In addition, a selective post-translational loss of the α-subunit was apparent in cerebellar granule cells, which indicates that the α-subunit is co-assembled with the α6-subunit. The absence of the α6-subunit triggered various additional changes in the cerebellum, which included a reduction in the affinity of the GABA<sub>ᵦ</sub> receptor for muscimol, an increase in the number of receptors containing the β3-subunit compared with wild-type and, interestingly, a compensatory upregulation of a K<sup>⁺</sup> channel (TASK-1) in granule cells. Surprisingly, the expression of genes encoding α1- and β2- (but not γ2-) subunits in the forebrain was reduced. This effect was presumably due to their colocalization with the gene encoding the α6-subunit in the same gene cluster and the absence of the knockout animals of the neomycin resistance cassette, which might alter the expression of neighboring genes. Thus, the behavioral phenotype of the α6-knockout mice might reflect the upregulation of a K<sup>⁺</sup> channel and the downregulation of GABA<sub>ᵦ</sub> receptor subunits other than α6. On this premise the phenotype has limited value for in vivo pharmacological analysis of defined GABA<sub>ᵦ</sub> receptor subtypes.

Targeting the gene encoding the β3-subunit
GABA<sub>ᵦ</sub> receptors that contain the β3-subunit are a prevalent receptor population present in most brain areas. Deletion of the gene encoding the β3-subunit results in mice that possess only half of the normal density of GABA<sub>ᵦ</sub> receptors in the brain. Most of these mice die in the neonatal period, however, a few survive and grow to normal body size, although these mice display various neurological impairments including hyperresponsiveness to sensory stimuli, strong motor impairment and epileptic seizures, which might be due to the lack of β3-containing receptors as ‘desynchronizers’ of neuronal activity. These three features are similar to clinical signs of Angelman syndrome. Pharmacologically, the effectiveness of pentobarbital, enflurane and halothane in impairing the righting reflex remained unaltered in β3-knockout mice, whereas midazolam and etomidate were less effective in this test, compared with wild-type mice. The immobilizing effect of enflurane and halothane was strongly impaired, which indicates that β3-containing receptors are important in the mediation of the immobilizing (tail clamp) but not in the mediation of the obtunding (loss of righting reflex) effects of the volatile anesthetics halothane and enflurane.

Targeting the gene encoding the δ-subunit
A small population of GABA<sub>ᵦ</sub> receptors that are present mainly in the cerebellum and thalamus are those that contain the δ-subunit. δ-Knockout mice displayed an attenuation of the sleep time following the administration of the neurosteroids alphaxalone and pregnanolone, whereas the response to propofol, etomidate, ketamine and midazolam was indistinguishable from that observed in wild-type mice. The latter finding is consistent with the absence of benzodiazepine sites in δ-containing receptors.

Targeting the gene encoding the γ2-subunit
A subtle strategy was used to assess the function of the large splice variant of the γ2-subunit. The γ2<sub>L</sub> cDNA contains an additional 24-bp exon, which provides a phosphorylation target sequence in the transmembrane (TM)3-TM4 loop. When the γ2<sub>L</sub>-specific exon is deleted from the genome and thus γ2<sub>L</sub> is converted to γ2<sub>S</sub>, the mutant animals display normal behavior. After treatment with midazolam and zolpidem but not pentobarbital and etomidate, the sleep time was slightly prolonged (+20%). However, in the γ2<sub>L</sub>-knockout mice, the γ2<sub>S</sub>-subunit variant was upregulated in whole mouse brain by ~2.4-fold. It is not clear whether the upregulation of γ2<sub>S</sub> influenced the benzodiazepine response.

Mice deficient in both the γ2<sub>S</sub>- and γ2<sub>L</sub>-subunits are entirely devoid of a response to benzodiazepines as shown behaviorally and in cultured dorsal root ganglion cells. Most homozygous γ2-knockout mice die perinatally. This is due, at least in part, to the requirement of the γ2-subunit for synaptic clustering of GABA<sub>ᵦ</sub> receptors, although not for receptor assembly. In animals that survive for up to two weeks, diazepam failed to induce sedation and to impair the righting reflex. This response failure reflects the requirement of the γ2-subunit for the formation of the benzodiazapine site of GABA<sub>ᵦ</sub> receptors. By contrast, mice heterozygous for the γ2-subunit knockout mutation develop and behave normally. The synaptic clustering of GABA<sub>ᵦ</sub> receptors is only partly reduced (~15–30%, depending on the brain region); the undamaged receptors consist of α- and β-subunits. When exposed to certain fear-inducing stimuli, these animals show a striking disease phenotype with a high anxiety response to natural and learned aversive stimuli, as
sensitive receptor subtypes (Fig. 1cortex). The four receptor subtypes are expressed in the olfactory bulb.

α-basal forebrain cholinergic neurons and the reticular nucleus of the thalamus express exclusively the α-containing GABAA receptors. Diazepam-sensitive GABAA receptor subtypes. HEK293 cells were transiently transfected with the cDNAs encoding wild-type α1, α2, α3 and α5-subunits or the mutated α1(H101R)-, α2(H101R)-, α3(H126R)- and α5(H105R)-subunits in combination with β2-(for α1 and α3) or β3-(for α2 and α3) and γ2-subunits. The modulation of the GABA-induced Cl– currents by 1 μM diazepam is expressed relative to the control currents recorded at the same GABA concentration (3 μM for α1, α3- and α5-containing receptors, and 30 μM for α2-containing receptors). Data shown are mean ± SE; n = 3–7 (Ref. 27).

Fig. 1. Immunohistochemical distribution of diazepam-sensitive GABAA receptor subtypes. Diazepam-sensitive receptor subtypes (α1, α2, α3, and α5-containing receptors) are attributed to largely distinct neuronal circuits, as demonstrated by the localization of the corresponding α-subunit variants in parasagittal sections of mouse brain. False color coding indicates different levels of α-subunit expression: white (high expression) > yellow > red > purple (low expression) > blue (no expression). The α1-containing GABAA receptors are most prevalent, particularly in the cerebral and cerebellar cortex. The α2-containing receptors are largely expressed in the hippocampus, amygdala (not visible) and striatum, whereas the monoaminergic and serotonergic neurons of the brain stem, α5-containing receptors are largely restricted to the hippocampus. All four receptor subtypes are expressed in the olfactory bulb.

well as a cognitive bias for threat cues. In patients with panic anxiety a partial reduction in the number of GABAA receptors has been shown by positron emission tomography (PET) imaging. This receptor deficit is therefore likely to contribute to the disease process.

Limitations of the knockout strategy
Although studies with knockout mice can provide important information on GABAA-receptor-mediated functions, some limitations exist. For example, the knockout mutation might trigger adaptive changes during development and in neuronal function, and such a complex phenotype makes it difficult to draw unbiased conclusions regarding the function of an individual GABAA receptor subtype. For β3- and γ2-knockout mice, the phenotype is largely lethal and the behavior of the few surviving animals might not necessarily be representative of the mutation but could rather reflect an accidental constellation of genetic and possibly environmental factors. Furthermore, all GABAA receptor knockouts published so far (and almost all knockouts in general) harbor a neomycin resistance cassette in the mutated gene. The presence of the neomycin resistance cassette (a positive selection marker in embryonic stem cells) might in some cases alter the expression of neighboring genes presumably via its own regulatory elements. Because genes encoding GABAA receptor subunits are clustered at the chromosomal level, the expression of neighboring genes encoding other GABAA receptor subunits might be affected by the neomycin resistance cassette in the knockout mouse. This is most probably the case for the gene encoding the α6-subunit, which is part of the α1, α6, β2, γ2 gene cluster. Although expression of the α6-subunit is limited to the cerebellum, the expression of the genes encoding α1- and β2-subunits was shown to be decreased in the forebrain of α6-knockout mice; presumably this was due to regulatory elements of the neomycin resistance cassette. Thus, although the knockout mice can provide interesting information on receptor assembly and function and on compensatory adaptations, these adaptations might largely preclude a meaningful molecular interpretation of drug responses with regard to a particular receptor. More sophisticated approaches are required to attribute pharmacological functions to GABAA receptor subtypes in vivo.
The term knock-in point mutation refers to the replacement of a single amino acid codon in a defined gene in vivo. Studies on recombinant GABA<sub>\alpha</sub> receptors had indicated that a His to Arg point mutation in the benzodiazepine binding site of GABA<sub>\alpha</sub> receptors abolished binding of classical benzodiazepines but it apparently did not affect receptor assembly and sensitivity to GABA (Refs 26,27). Thus, the corresponding knock-in point mutation is not expected to be susceptible to appreciable changes in brain development or function. Knock-in point mutations were therefore chosen as a strategy to dissect the pharmacology of GABA<sub>\alpha</sub> receptor subtypes.

**A molecular switch in vivo**

The functional dissection of GABA<sub>\alpha</sub> receptor subtypes was achieved by focusing on the benzodiazepine sites as a distinctive feature. The vast majority of GABA<sub>\alpha</sub> receptors contain a binding site for diazepam and other related classical benzodiazepines that is located at the interface of the \( \gamma \)-2 subunit and the respective \( \alpha \)-subunit (either \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \) or \( \alpha_5 \)) (Fig. 1). These \( \alpha \)-subunits contain a common feature: a conserved histidine residue in the drug-binding domain. Its conversion to an arginine residue renders the respective receptor diazepam-insensitive in vitro<sup>26,27</sup> (Fig. 2). Exploiting this molecular switch, the His to Arg point mutation was introduced into the germ line of mice in the genes that encode the \( \alpha_1 \), \( \alpha_2 \) and \( \alpha_3 \)-subunits (\( \alpha_1 \) (H101R), \( \alpha_2 \) (H101R), \( \alpha_3 \) (H126R)). These mouse lines were received an inescapable shock 24 h previously was measured.

**Distribution of diazepam-insensitive binding sites**

Corresponding to that of wild-type receptors as shown for \( \alpha_1 \), \( \alpha_2 \) and \( \alpha_3 \)-containing point-mutated receptors (Fig. 3). Most importantly, the physiology of neuronal circuits appeared to be unaffected in the point-mutated mouse lines. The GABA-induced responses of the mutated receptors were unaltered in cells expressing the \( \alpha_1 \) (H101R)-containing receptor<sup>29</sup> or the \( \alpha_2 \) (H101R)-containing receptor<sup>29</sup>, which indicates that the operation of the GABA-gated ion channels by the physiological ligand had remained unchanged. Only the affinity for diazepam was reduced – by a factor of at least 300 (Refs 29,30). The point-mutated mice showed no overt distinctive phenotype. Behaviorally, the pharmacological responses to diazepam and other benzodiazepine site ligands were selectively attenuated.

Some GABA<sub>\alpha</sub> receptors contain two types of \( \alpha \)-subunits. However, it is currently not known how the presence of a point-mutated \( \alpha \)-subunit in combination with another type of \( \alpha \)-subunit would affect the regulation of GABA-induced Cl<sup>-</sup> currents by benzodiazepines.

### Table 1. Benzodiazepine pharmacology of GABA<sub>\alpha</sub> receptor subtypes

<table>
<thead>
<tr>
<th>Pharmacological effect</th>
<th>Receptor involved</th>
<th>Refs</th>
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<tbody>
<tr>
<td>Anxiolysis</td>
<td>( \alpha_2 )-containing</td>
<td>30</td>
</tr>
<tr>
<td>Sedation</td>
<td>( \alpha_1 )-containing</td>
<td>29,32</td>
</tr>
<tr>
<td>Anticonvulsion</td>
<td>( \alpha_1 )-containing and those not containing ( \alpha_1 )</td>
<td>29,32</td>
</tr>
<tr>
<td>Anterograde amnesia</td>
<td>( \alpha_1 )-containing</td>
<td>29</td>
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*Anxiolysis was measured as the drug-induced reduction of a behavioral avoidance response (light-dark choice test and elevated plus maze test). Sedation was measured as a decrease of motor or locomotor activity. Anticonvulsant activity was tested as the attenuation of pentylenetetrazole-induced convulsions. Anterograde amnesia was determined in a passive avoidance paradigm; the latency to re-enter a dark compartment in which the animal had received an inescapable shock 24 h previously was measured.*
α1-mutant animals29. Therefore, these actions are most probably mediated by GABA_A receptors that contain α-subunits other than the α1-subunit.

Using a strategy identical to that outlined above29, McKernan et al. independently generated α1(H101R) mice32. In both reports, the point mutation was introduced by a targeting vector carrying a neomycin resistance marker flanked by loxP-sites. This marker was deleted by cre-mediated recombination. McKernan et al.32 reported certain findings from behavioral studies that apparently differed somewhat from those reported by Rudolph et al.29 on α1(H101R) mice. However, these differences were largely due to methodological differences in the behavioral test procedures used33. It is likely that the diazepam-induced increase of motor activity in α1(H101R) mice but not in wild-type mice described by McKernan et al. was because the motor activity was measured in an unfamiliar (‘stressful’) environment32. Under these conditions, diazepam did not decrease motor activity even in wild-type mice32. In the report by Rudolph et al., the mice spent at least 24 h in the test cage located in the test room before the horizontal motor activity was measured33. In this set-up, diazepam decreased the horizontal motor activity in wild-type mice but not in α1(H101R) mice. However, if mice were transferred to a new room 30 min before drug treatment, diazepam had no apparent effect on wild-type mice but increased the motor activity of α1(H101R) mice33. This is similar to the findings of McKernan et al.32 Thus, the novelty or familiarity of the test environment appears to be a crucial parameter. Furthermore, the rotarod test was performed by the two groups at different speeds. At a low speed, diazepam decreased the latency to fall off the rotating rod similarly in wild-type mice and α1(H101R) mice29. When McKernan et al. tested trained animals at a high rotating speed, diazepam had no effect on the latency to fall off the rod in α1(H101R) mice at doses up to 10 mg kg\(^{-1}\), whereas the latency in wild-type mice was decreased at the same dose32. When Crestani et al.33 used a high-speed version rotarod test (4–40 rpm, ten steps of 25 s each), they also observed that 10 mg kg\(^{-1}\) diazepam decreased the latency to fall off the rod in wild-type mice but not in α1(H101R) mice. Thus, under comparable test conditions the responses of the α1(H101R) mice from the two groups are similar. The rotarod test was not suitable to assess selectively diazepam-induced ataxia because at the dose of 10 mg kg\(^{-1}\) the wild-type mice are strongly sedated. The presence or absence of anxiolytic activity of diazepam in the α1(H101R) mice was not reported on by McKernan et al.32

**Fig. 4.** Making the light–dark choice. The anxiolytic effect of diazepam (Valium\(^{TM}\)) is apparent in the wild-type mice (WT) by the time spent in the lit area. In the α2(H101R) mutant mice (α2 MUT), the α2-containing GABA_A receptor is rendered diazepam insensitive by a point mutation. Diazepam fails to display an anxiolytic effect as shown by the avoidance of the lit area by the mutant. These results demonstrate that the anxiolytic action of diazepam in wild-type mice is mediated by α2-containing GABA_A receptors.

Anxiolytic activity is mediated by α2-containing receptors

Novel anxiolytic agents that largely lack sedative components and, in particular, dependence liability are much sought after. It would therefore be important to determine whether the anxiolytic activity of diazepam could be attributed to neuronal circuits characterized by a particular GABA_A receptor subtype. This was recently accomplished on the basis...
of the analysis of α2- and α3-point-mutated mouse lines containing the Hist Arg point mutation in the respective benzodiazepine binding site. Neurons that express exclusively α3-containing receptors are located in the reticular activating system (i.e. noradrenergic, dopaminergic, and serotonergic neurons) and in the basal forebrain (cholinergic neurons). Previously, it had been suggested that the anxiolytic effect of diazepam is due to the dampening in particular of the noradrenergic neurons in the locus coeruleus and its interactions with serotonergic neurons. However, in α2(H126R) mice the anxiolytic activity of diazepam, as tested by the light–dark choice test and the elevated plus-maze test, was not impaired compared with wild-type mice. By contrast, in α2(H101R) mice the anxiolytic activity of diazepam was absent (Fig. 4). Thus, the anxiolytic activity of diazepam is considered to be mediated by neurons expressing α2-containing receptors (Table 1). This finding is consistent with the expression of α2 subunits in brain areas that are associated with emotional stimulus processing. The central nucleus of the amygdala contains mainly α2-containing receptors. In addition, α2-containing receptors are densely packed on the axon initial segment of principal cells of the cerebral cortex and the hippocampus bringing their output activity under GABA-mediated control. Given that α2-containing receptors constitute only ~15% of diazepam-sensitive GABA_A receptors, ligands selective for α2-containing GABA_A receptors would be expected to show a much reduced side-effect profile. Such agents would be highly selective drugs compared with the nonselective benzodiazepines in clinical use.

### Subtype-selective ligands of the benzodiazepine site

These new insights into the subtype specificity of benzodiazepine actions provide precise guidelines for the development of novel drugs with more selective actions and fewer side-effects than those currently in clinical use. A major factor in anxiolytic profiling is the avoidance of a response at α1-containing receptors in favor of α2-, α3-, and α5-containing receptors. The novel ligand L838417, developed and characterized by McKernan et al., is a breakthrough in this direction. L838417 binds with high affinity to α1-, α2-, α3- and α5-containing receptors, but not to α4- or α6-containing receptors, and it is similar in this respect to diazepam. However, in contrast to diazepam, which is a full agonist at all benzodiazepine-sensitive GABA_A receptors, L838417 fails to enhance the GABA response at α1-containing receptors but acts on α2-, α3- and α5-containing receptors apparently with partial agonistic activity. Behaviorally, L838417 enhanced the exploration of a novel environment in a locomotor activity test equally in wild-type and α1(H101R) mice, which suggests that this in vivo activity was mediated by α2-, α3- and/or α5-containing receptors. L838417 possessed anticonvulsant activity but showed no effect on rotorod performance in mice. In the operant chain-pulling test in rats, 10 mg kg^-1 diazepam impaired the performance, whereas L838417 at doses of ≤30 mg kg^-1 was without effect. L838417 displayed anxiolytic-like activity in wild-type rats as shown in the elevated plus-maze test and in a conditioned fear-potentiated startle protocol. The behavioral characterization of L838417 supports the conclusion that the sedative but not the anxiolytic-like properties of benzodiazepines are mediated by α1-containing GABA_A receptors. It is expected that more advanced anxiolytic drugs would be agonists selective for α2-containing GABA_A receptors.

Other ligands that have some degree of GABA_A receptor selectivity – apart from the classical ligands zolpidem and abecarnil - include zaleplon and SL651498. Zaleplon is a ligand with preferential affinity for α1-containing receptors compared with α2-, α3- and α5-containing receptors. This affinity pattern is consistent with its profile as a hypnotic. SL651498 displays a comparable affinity for α1, α2- and α3-containing receptors but differentiates these receptors by its intrinsic activity as a full agonist at α2- and α3-containing receptors and as a partial agonist at α1-containing receptors; it also has potent anxiolytic activity in animal models. These examples illustrate the ongoing attempts to direct the search for novel ligands of the benzodiazepine site towards specific receptor subtypes.

### Concluding remarks

Targeting drugs to GABA_A receptor subtypes holds the promise of increased clinical specificity compared with the classical benzodiazepines, which act indiscriminately on all diazepam-sensitive GABA_A receptors. In addition, subtype-selective drugs are expected to display fewer side-effects, such as tolerance and dependence liability, because they affect only a small population of GABA_A receptors. Furthermore, subtype-specific ligands might be useful for the treatment of neuropsychiatric disorders beyond the classical spectrum of benzodiazepines. Finally, the subtype specificity of drug actions is unlikely to be restricted to ligands of the benzodiazepine site and is expected to extend to other types of GABA_A receptor modulators. Thus, the strategy of generating point-mutated knock-in mice has accelerated the recognition of the role of individual GABA_A receptor subtypes as drug targets.
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Chemical name

Ro154513: ethyl 8-iodo-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate

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