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ANALYSIS OF $GABA_A$ RECEPTOR FUNCTION AND DISSECTION OF THE PHARMACOLOGY OF BENZODIAZEPINES AND GENERAL ANESTHETICS THROUGH MOUSE GENETICS

Uwe Rudolph and Hanns Möhler
Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich; email: rudolph@pharma.unizh.ch, mohler@pharma.unizh.ch

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■ **Abstract** $GABA_A$ receptors are molecular substrates for the regulation of vigilance, anxiety, muscle tension, epileptogenic activity, and memory functions, and the enhancement of $GABA_A$ receptor-mediated fast synaptic inhibition is the basis for the pharmacotherapy of various neurological and psychiatric disorders. Two kinds of $GABA_A$ receptor-targeted mutant mice have been generated: (*a*) knockout mice that lack individual $GABA_A$ receptor subunits ($\alpha1$, $\alpha5$, $\alpha6$, $\beta2$, $\beta3$, $\gamma2$, δ, and $\rho1$) and (*b*) knockin mice that carry point mutations affecting the action of modulatory drugs [$\alpha1$(H101R), $\alpha2$(H101R), $\alpha3$(H126R), $\alpha5$(H105R), and $\beta3$(N265M)]. Whereas the knockout mice have provided information primarily with respect to the regulation of subunit gene transcription, receptor assembly, and some physiological functions of individual receptor subtypes, the point-mutated knockin mice in which specific $GABA_A$ receptor subtypes are insensitive to diazepam or some general anesthetics have revealed the specific contribution of individual receptor subtypes to the pharmacological spectrum of diazepam and general anesthetics.

INTRODUCTION

The dynamics of neural networks are largely shaped by the activity pattern of interneurons, most of which are GABAergic (1–6). Different patterns of rhythmic activity, including theta (4–12 Hz), gamma (30–100 Hz), and fast (>200 Hz) oscillations, which involve the synchronous firing of principal neurons and interneurons, subserve many functions in the developing and adult CNS. Cortical interneuron networks may generate both slow and fast cortical oscillatory activity (7–13). Similarly, inhibitory neurons of the thalamic reticular and perigeniculate nuclei generate the synchronized activity of thalamocortical networks (14). Gamma oscillations (30–100 Hz) occur in various brain structures (15–17) and can occur over large distances. They could, therefore, provide a substrate for

"binding" together spatially separated areas of cortex, a hypothetical process whereby disparate aspects of a complex object, for example, are combined to form a unitary perception of it (17, 18). Furthermore, the activity of the interneurons is thought to set the spatiotemporal conditions required for synaptic plasticity and hippocampus-dependent learning (2, 4, 6, 19–23).

GABAergic interneurons are morphologically highly diverse and display two striking features: (*a*) They innervate the principal cells in a domain-specific manner. Thus, particular input signals to principal cells can be regulated selectively via specific interneurons. Similarly, the output of principal cells can be specifically regulated owing to the presence of interneurons with selective axo-axonic innervation. (*b*) The response properties of interneuron signaling are determined by the type of $GABA_A$ receptor expressed in the respective domain of the principal cell. For instance, the soma of hippocampal pyramidal cells is innervated by two types of basket cells. The fast spiking parvalbumin-containing basket cells form synapses that contain $\alpha 1$ $GABA_A$ receptors. These receptors are endowed with fast kinetics of deactivation and desensitisation (3, 5, 24, 25). In contrast, the regular spiking CCK-positive basket cells form synapses contain α_2 $GABA_A$ receptors (26–29). The α_2-containing receptors display slower kinetics than α_1-containing receptors. α_2-Containing $GABA_A$ receptors also mediate simple on/off signaling as shown by their presence on the axon-initial segment of principal cells. Thus, functionally specialized interneurons operate in conjunction with the kinetically appropriate $GABA_A$ receptor subtype. Because GABAergic interneurons are operative throughout the brain, a highly diverse repertoire of $GABA_A$ receptors is required to set the tempo-spatial pattern of neuronal networks. A mutational analysis of $GABA_A$ receptor subtypes therefore promises to advance the understanding of brain function in three ways: (*a*) The physiological significance of $GABA_A$ receptor subtypes will be revealed; (*b*) based on the expression pattern of $GABA_A$ receptors, the contribution of particular subsets of neurons to the regulation of neuronal networks and behavior becomes apparent; and (*c*) the pharmacological significance of $GABA_A$ receptor subtypes is identified, providing new avenues for drug development (see also Figure 1 for a schematic representation of drug-binding sites on the $GABA_A$ receptor).

$GABA_A$ RECEPTOR EXPRESSION AND ASSEMBLY IN MICE LACKING INDIVIDUAL $GABA_A$ RECEPTOR SUBUNITS

Genetic ablation of individual $GABA_A$ receptor subunits has allowed us to address the question of whether the knocked-out subunit is replaced by another subunit and whether compensatory changes in the expression of other $GABA_A$ receptor subunits or other receptors or channels occur. The answers to these questions are important when interpreting, e.g., behavioral phenotypes in knockout mice.

The α subunits that have been knocked out so far are $\alpha 1$, $\alpha 5$, and $\alpha 6$. In the $\alpha 1$ knockout mice, there is a loss of more than 50% of all $GABA_A$ receptors (30).

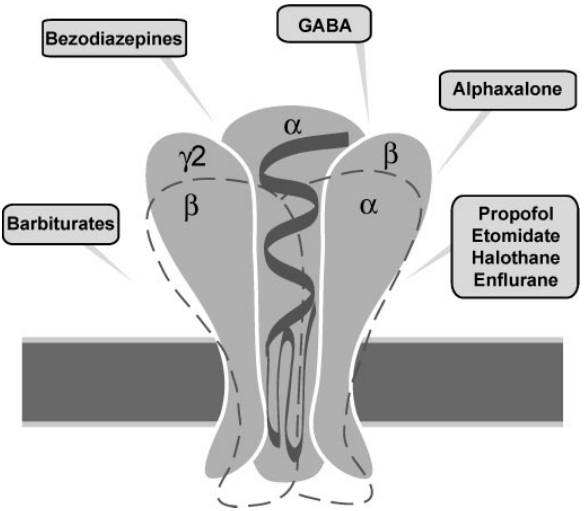

Figure 1 Scheme of the $GABA_A$ receptor and its major drug-binding sites. Two of the five subunits are depicted as being translucent. Most $GABA_A$ receptors are composed of α, β, and $\gamma 2$ subunits. Each subunit polypeptide chain has a large N-terminal portion, four transmembrane regions, and a short C-terminal extracellular portion, as outlined by the black ribbon. Amino acid residues of the α and β subunits contribute the binding site for GABA, whereas residues of the α and γ subunits contribute to the binding site for benzodiazepines. Amino acid residues of the α and β subunits are critical for the action of halothane and enflurane with the same amino acid residues in the β subunit being also critical for the action of etomidate and propofol. The binding site for barbiturates involves, at least in part, residues of the β subunit. The binding site for neurosteroids, such as alphaxalone, is molecularly not yet defined.

The upregulation of the $\alpha 2$ and $\alpha 3$ subunits by 37% and 39%, respectively (30, 31), the downregulation of $\beta 2/3$ by 65% and $\gamma 2$ subunits by 47%(31), and the downregulation of the $\alpha 6$ subunit in the cerebellum by 38% (30) most likely represent compensatory changes that have to be considered when interpreting the other phenotypes. In the $\alpha 5$ knockout mice, the number of benzodiazpine-binding sites in the hippocampus is decreased by approximately 16%, which corresponds to the number of $\alpha 5$ receptors in these brain regions and suggests no upregulation of other α subunits (32). When examining mice that have deleted a chromosomal region including the $\alpha 5$ gene, it was found that the expression of $\beta 2/3$ and $\gamma 2$ subunits were decreased exclusively in neurons expressing the $\alpha 5$ subunit in wild-type mice, indicating that the assembly of the entire receptor complex is prevented (33). Furthermore, the subcellular distribution of the $\alpha 2$ subunit was unchanged in these mice (34). In $\alpha 6$ knockout mice, the $\beta 2$, $\beta 3$, and $\gamma 2$ subunits were reduced by approximately 50%, 20%, and 40% in the cerebellum (where the $\alpha 6$ subunit is almost exclusively expressed), so that the proportion of receptors

containing the $\beta 3$ subunit was increased, indicating that the remaining $GABA_A$ receptors have a modified subunit distribution compared to wild type (35). In the forebrain, where the $\alpha 6$ subunit is not expressed, the expression of the $\alpha 1$ and $\beta 2$ subunits was reduced by 43% and 25%, respectively. The most likely explanation is that the neomycine resistance cassette that has been inserted into the $\alpha 6$ subunit gene downregulates the expression of the neighboring $\alpha 1$ and $\beta 2$ genes (36). These changes in subunit expression described above may limit the usefulness of the $\alpha 6$ knockout model. In the $\alpha 6$ knockout mice, δ subunit–specific antibodies almost fail to recognize the δ subunit despite the δ subunit mRNA being present, indicating a posttranslational loss of δ and thus a partnership between the $\alpha 6$ and δ proteins (37).

Lack of the $\beta 2$ or the $\beta 3$ subunit leads to dramatic changes in the expression of other receptor subunits. In $\beta 2$ knockout mice, expression of the α subunits $\alpha 1$–$\alpha 6$ was reduced by approximately 39%–69%, indicating that it substantially associates with all known α subunits and that other β subunits do not substitute for the missing $\beta 2$ subunit (30). Similarly, in the $\beta 3$ knockout mice, approximately half of the $GABA_A$ receptors were lost (38). Thus, the lack of a β subunit appears to prevent the formation of the respective receptors.

The $\gamma 2$ subunit is by far the most abundant γ subunit. In $\gamma 2$ knockout mice, which die shortly after birth, the number of $GABA_A$ receptors was apparently unchanged, whereas the number of benzodiazepine-binding sites was reduced by approximately 90%, consistent with the $\gamma 2$ subunits being necessary for benzodiazepine binding (39). However, the $\alpha\beta$ channels in these mice displayed unusual behavior: The single-channel conductance was reduced (39, 40) and the receptors were not properly clustered (41). Nevertheless, $\alpha\beta$ receptors are assembled and inserted into the plasma membrane in the absence of the $\gamma 2$ subunit.

In forebrains of δ subunit knockout mice, the amount of the $\alpha 4$ subunit, which normally associates with δ, was decreased, whereas the amount of the $\gamma 2$ subunit was increased (42, 43). Because the amount of $\alpha 4$ subunit immunoprecipitated by a $\gamma 2$ antibody was increased, the remaining $\alpha 4$ subunits appear to be more often associated with $\gamma 2$ subunits (42). The changes were largely confined to brain regions normally expressing the δ subunit (43). Thus, the δ subunit appears to interfere with the coassembly of $\alpha 4$ and $\gamma 2$ subunits.

These findings demonstrate that the knockout of individual $GABA_A$ receptor subunits may lead to secondary changes with respect to formation of receptors and to compensatory upregulation, thus increasing the complexity of the phenotypes.

PHYSIOLOGICAL, PHARMACOLOGICAL, AND BEHAVIORAL STUDIES OF $GABA_A$ RECEPTOR FUNCTION IN $GABA_A$ RECEPTOR SUBUNIT KNOCKOUT MICE

In the following paragraph, we review the lessons learned from gene knockout mice with a particular emphasis on behavioral phenotypes.

The following $GABA_A$ receptor subunits have been knocked out to date: $\alpha 1$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, $\gamma 2$, δ, and $\rho 1$. Selected phenotypes of these mice are summarized in Table 1.

$\alpha 1$ Subunit Knockout Mice

Lines of $\alpha 1$ knockout mice have been generated in two laboratories (29, 30). Because the majority of diazepam-sensitive $GABA_A$ receptors contain the $\alpha 1$ subunit, it is somewhat surprising that $\alpha 1$ knockout mice are viable, although underrepresented, in offspring from heterozygote crosses, indicating that there is some lethality. Apart from the changes in subunit expression described above, $\alpha 1$ knockout mice had lower body weights (approximately 30%) until the age of at least 3 months and exhibited a tremor when handled (30, 31). The developmental changes of inhibitory synaptic currents in cerebellar neurons seen in wild-type mice were absent (29) and the hippocampal miniature inhibitory postsynaptic currents (IPSCs) were prolonged (44). In line with expectations, all high-affinity [^{3}H]zolpidem-binding sites were lacking in the $\alpha 1$ knockout mice (31). The $\alpha 1$ knockout mice did not display any major deficits in beam balancing and swimming ability tests (30). The level of spontaneous locomotor activity and exploration and the performance on the rotating rod were similar to wild type (30). The biccuculine-induced seizure susceptibility was increased (31). Surprisingly, $\alpha 1$ knockout mice were more sensitive to the motor-impairing/sedative effects of diazepam, determined as decrease in locomotor activity (45, 46). This is in contrast to the observation that diazepam does not reduce horizontal motor activity in $\alpha 1$(H101R) mice carrying diazepam-insensitive $\alpha 1$-containing $GABA_A$ receptors [see also $\alpha 1$(H101R) Knockin Mice, below] (47). The reason for this discrepancy might be that compensatory changes in the $\alpha 1$ knockout mice may be caused by the lack of the subunit throughout development, which leads to a significant change in the functions of other $GABA_A$ receptors. The anxiolytic-like action of diazepam in the elevated plus maze was still present in the $\alpha 1$ knockout mice (45), consistent with earlier observations that the anxiolytic-like action of diazepam is retained in $\alpha 1$(H101R) mice (47). This supports the notion that $GABA_A$ receptors containing α subunits other than $\alpha 1$ are mediating the anxiolytic-like action of diazepam in mice. The hypnotic effect of a high dose of diazepam, measured as the duration of the loss of the righting reflex, was increased in $\alpha 1$ knockout mice compared to wild type, whereas the hypnotic effect of a high dose of zolpidem was reduced in $\alpha 1$ knockout mice compared to wild type (45, 48). Because high-affinity sites for zolpidem are absent in the $\alpha 1$ knockout mice and zolpidem has no affinity for $GABA_A$ receptors containing the $\alpha 5$ subunit, its action on the $\alpha 1$ knockout mice must be mediated by $\alpha 2$- or $\alpha 3$-containing $GABA_A$ receptors, which are upregulated in the $\alpha 1$ knockout mice. Diazepam has a significantly higher affinity to $\alpha 2$- or $\alpha 3$-containing $GABA_A$ receptors than zolpidem and also a high affinity to $\alpha 5$-containing $GABA_A$ receptors, and, surprisingly, its hypnotic action is increased in the $\alpha 1$ knockout mice. Comparable experiments have not been reported for

TABLE 1 Overview of selected phenotypes of $GABA_A$ receptor subunit knockout (KO) and knockin (KI) mice

$\alpha 1$	KI: Mediation of sedative action of diazepam KI: Mediation of anterograde amnesic action of diazepam KI: Mediation of anticonvulsant action of diazepam (partial) KO: Reduction of body weight (30%) KO: No spontaneous seizures, but seizure susceptibility increased KO: Tremor KO: Normal locomotor activity and motor performance	(29, 30, 47, 79)
$\alpha 2$	KI: Mediation of anxiolytic action of diazepam KI: Mediation of myorelaxant action of diazepam (partial)	(80, 87)
$\alpha 3$	KI: Mediation of myorelaxant action of diazepam (partial)	(80, 87)
$\alpha 5$	KO: Improved performance in a hippocampus-dependent task (spatial learning) KI: Facilitation of trace fear conditioning KI: Mediation of myorelaxant action of diazepam (partial)	(32, 49)
$\alpha 6$	KO: Posttranslational loss of δ subunit in cerebellum KO: increased expression of TASK-1 K^+ channels	(37, 53)
$\beta 2$	KO: Increased locomotor activity in novel environment KO: No spontaneous seizures	(30)
$\beta 3$	KO: Cleft palate KO: Neonatally lethal (ca. 90%) KO: Hyperactive KO: Spontaneous seizures KO: Hyperresponsive KO: Motor impairment KI: Immobilizing action of etomidate and propofol absent KO, KI: Immobilizing action of halothane and enflurane: diminished potency	(38, 57, 59, 99)
$\gamma 2$	KO-Homozygotes: Neonatally lethal Defects in postsynaptic clustering of $GABA_A$ receptors KO-Heterozygotes: Reduction of synaptic clustering, e.g., in hippocampus Chronic anxiety Heightened responsiveness in trace fear conditioning and ambiguous cue discrimination	(39, 41, 65)
δ	KO: Attenuation of responses to neuroactive steroids KO: Reduced ethanol consumption KO: Attenuated withdrawal from chronic ethanol exposure KO: Reduced anticonvulsant effect of ethanol	(70, 102)
$\rho 1$	KO: Alteration of the excitation/inhibition balance between second and third retinal neurons	(76)

α1(H101R) mice. The hypnotic effects of the barbiturate pentobarbital were similar in α1 knockout and wild-type mice, whereas the hypnotic effect of ethanol was decreased in mutant males but not in mutant females (48). Another set of experiments suggests that compensation may also have occurred outside of the $GABA_A$ receptor system. Whereas the number of D1, D2, and NMDA receptors, the prepulse inhibition of acoustic startle, and the effects of cocaine on conditioned place preference were similar in α1 knockout and wild-type mice, amphetamine and cocaine induced stereotypy instead of hyperlocomotion in α1 knockout mice (46).

α5 Subunit Knockout Mice

The α5 subunit is expressed primarily in hippocampus, including the CA1 and CA3 regions. α5 Subunit knockout mice display normal motor performance and coordination (32). The benzodiazepine chlordiazepoxide retained its anxiolytic-like action in these mice, indicating that α5-containing $GABA_A$ receptors are not involved in this action (32). In the Morris water maze test, a spatial learning task dependent on hippocampal function, α5 knockout mice displayed a decreased latency to find the submerged platform compared to wild-type mice, indicating that these mice show a significantly improved performance (32). Evidence for the specific alteration of hippocampal functions were also obtained in point-mutated α5(H105R) mice (49) [see also α5(H105R) Knockin Mice, below]. The amplitude of the IPSCs was decreased in the CA1 region of hippocampal slices from α5 knockout mice and the paired-pulse facilitation of field EPSP (fEPSP) amplitudes were enhanced (32). Thus, results obtained with the α5 knockout mice suggested that an inverse agonist selective for α5-containing $GABA_A$ receptors may be suitable as a drug-enhancing cognitive function (32). Indeed, a compound [6,6-dimethyl-3-(2-hydroxyethyl)thio-1-(thiazol-2-yl)-6,7,-dihydro-2-benzothiophen-4(5H)-one, also called compound 43] has been identified, which has an approximately tenfold selectivity for α5-containing receptors over other $GABA_A$ receptors and is a benzodiazepine site inverse agonist at α5-containing $GABA_A$ receptors. It enhances the performance of rats in the delayed "Matching-to-place" Morris water maze test without any signs of convulsant or proconvulsant activity (50).

α6 Subunit Knockout Mice

The expression of the α6 subunit is largely restricted to the cerebellum. α6 Knockout mice showed the same level of exploratory activity in the open field as wild-type mice and learned the horizontal wire task (37). However, in the rotating rod test, α6 knockout mice were significantly more impaired by diazepam than wild-type mice (51), which is somewhat difficult to explain because the α6-containing $GABA_A$ receptors are insensitive to diazepam. The impairment of the rotarod performance by ethanol was similar in α6 knockout and wild-type mice (51). In another study, the duration of the loss of the righting reflex in response to ethanol, enflurane, and halothane was also unaltered, as was the tail-clamp/withdrawal response to

enflurane (52). These results suggest that the α6-containing $GABA_A$ receptors are not involved in these behavioral responses.

In cerebellar granule cells from α6 knockout mice, a tonic conductance, which is dependent on the presence of the $GABA_A$ receptor α6 subunit, is absent. The fact that the response of these cells to excitatory synaptic input is unaltered is likely due to an upregulation of the two-pore-domain K^+ channel TASK-1 (53).

β2 Subunit Knockout Mice

Because the β2 subunit is the most abundant of the three β subunits, it is somewhat surprising that the β2 knockout mice had normal body weights and did not display major deficits in the rotating rod, beam balancing, and swimming ability tests. In a novel environment, they exhibited a higher level of locomotor activity than wild-type mice, although they habituated to a similar degree as wild-type mice. The basis for this observation is not known (30).

β3 Subunit Knockout Mice

Before the advent of gene targeting technology, radiation-induced mutants were identified that lacked a chromosomal region including the α5, β3, and γ3 subunit genes, displayed a cleft palate, and died shortly after birth (54, 55). The fact that this phenotype was rescued with a β3 transgene strongly suggested that the lack of the β3 subunit gene was responsible for cleft palate (56).

When the β3 subunit was knocked out by gene targeting, approximately 57% of the mice developed cleft palate; however, 90% of the mice (i.e., for unknown reasons also most mice without a cleft palate) died within 24 h of birth. The partial penetrance of the cleft palate phenotype in the knockouts may reflect differences in genetic background (129/SvJ $\times$ C57BL/6J hybrid background in the case of the knockouts) or the contribution of other genes deleted in the radiation-induced mutants. Surviving β3 knockout mice are runted until weaning but achieve a normal body weight in adulthood (38). They display features reminiscent of Angelman syndrome in humans, including hyperactivity, poor learning and memory, poor motor coordination, repetitive stereotypical behavior (running continuously in tight circles), seizures, and EEG abnormalities (57). Thus, at least some clinical features of Angelman syndrome in humans may be related to a potentially impaired expression of the β3 subunit. Further phenotypic abnormalities of β3 knockout mice that have been described include an enhanced responsiveness to low-intensity thermal stimuli and a lack of antinociceptive activity of the $GABA_A$ receptor agonist THIP (58). It is, however, impossible to interpret these results as demonstrating an involvement of β3-containing $GABA_A$ receptors in this response because the antinociceptive effect of the $GABA_B$ receptor agonist, baclofen, was also reduced in β3 knockout mice, indicating that another neurotransmitter system unrelated to the $GABA_A$ receptor is also, most likely secondarily, affected by knockout of the β3 subunit (58). The duration of the loss of the righting reflex in response to midazolam and etomidate, but not to pentobarbital, ethanol, enflurane,

and halothane, was reduced in $\beta 3$ knockout mice, and the immobilizing action of enflurane and halothane as determined in a tail-clamp/withdrawal assay was decreased in $\beta 3$ knockout mice (59). This indicates that $\beta 3$-containing $GABA_A$ receptors are involved in mediation of this response to selected general anesthetics. Interestingly, in electrophysiological studies on the spinal cord, the sensitivity of evoked responses to enflurane did not differ between $\beta 3$ knockout and wild-type mice, which may be due to developmental and/or adaptive compensatory changes secondary to the knockout (60).

It was also observed that locomotor stimulation induced by cocaine was greater in $\beta 3$ knockout compared to wild-type mice; the mechanisms underlying this effect are not known (61). The sleep-enhancing effect of the endogenous unsaturated fatty acid amide oleamide were absent in $\beta 3$ knockout mice, indicating that oleamide mediates its sleep effects via $\beta 3$-containing $GABA_A$ receptors (62). The $\beta 3$ subunit is probably the only β subunit expressed in the reticular nucleus of the thalamus. In slices from $\beta 3$ knockout mice, GABA-mediated inhibition was abolished in reticular nucleus, but unaffected in relay cells. Oscillatory synchrony was dramatically intensified, suggesting that recurrent inhibitory connections in the reticular nucleus, which depend on $\beta 3$-containing $GABA_A$ receptors within the reticular nucleus, act as "desynchronizers" (63).

$\gamma 2$ Subunit Knockout Mice

Almost all $\gamma 2$ knockout mice die in the first few days after birth (39). They displayed a defect in postsynaptic clustering of $GABA_A$ receptors (41). The few animals that survived up to 18 days exhibited increased body and limb movements following birth, and later impaired grasping and righting reflexes and an abnormal gait (39). As already mentioned above, the single channel conductance of $GABA_A$ receptors that normally contains the $\gamma 2$ subunit was decreased (40). This decreased channel conductance and/or lack of postsynaptic clustering may be responsible for or at least contribute to the lethality of the phenotype. When the $\gamma 3$ subunit was expressed ectopically in the $\gamma 2$ knockout background, synaptic clustering was largely restored. However, this did not rescue the lethal $\gamma 2$ knockout phenotype (64).

Mice heterozygous for the $\gamma 2$ null mutation, $\gamma 2+/-$ mice, were viable and fertile. The synaptic clustering was reduced, mainly in hippocampus and cerebral cortex (65). These mice displayed an enhanced behavioral inhibition towards natural aversive stimuli and heightened responsiveness in trace fear conditioning and ambiguous cue discrimination learning. Thus, these mice represent a model of chronic anxiety, suggesting that $GABA_A$ receptor dysfunction may underlay a predisposition of patients to anxiety disorders. The anxiety-related behavioral inhibition of $\gamma 2$ heterozygous mice was reversed by diazepam, which is in line with the observation in humans that subjects with high anxiety scores are more sensitive to the anxiolytic actions of benzodiazepines than controls (65).

There are two splice variants of the $\gamma 2$ subunit, $\gamma 2$S and $\gamma 2$L, respectively, which arise by alternative splicing. $\gamma 2$S differs from $\gamma 2$L by the inclusion of an exon coding for eight additional amino acids. These amino acids are located in the third intracellular loop and include a consensus sequence for phosphorylation by protein kinase C. Previous work has suggested that these eight $\gamma 2$L-specific amino acids may be required for the potentiating action of ethanol at the $GABA_A$ receptor (66). However, $\gamma 2$L knockout mice displayed electrophysiological responses to ethanol indistinguishable from wild type, and the behavioral effects of ethanol, such as loss of the righting reflex, anxiolytic-like effect, acute functional tolerance, chronic withdrawal hyperexcitability, and hyperlocomotor activity, were not different from wild type. Thus, the $\gamma 2$L-specific exon appears not to be required for the modulatory action of ethanol at $GABA_A$ receptors and its behavioral effects (67). Furthermore, the affinity of brain membranes from $\gamma 2$L-specific knockout mice to benzodiazepine site agonists was increased, whereas the affinity of these brain membranes to benzodiazepine site inverse agonists (βCCM and Ro15-4513) was decreased. This finding would be compatible with the notion that the lack of the $\gamma 2$L subunits may shift the $GABA_A$ receptor from an inverse agonist-preferring configuration toward an agonist-preferring configuration (68). At the behavioral level, the hypnotic action (loss of righting reflex) of midazolam and zolpidem was increased (68). In a separate set of experiments, transgenic overexpression of $\gamma 2$S or $\gamma 2$L rescued the lethal $\gamma 2$ knockout phenotype, indicating that both forms are functionally equivalent in this respect (69).

δ Subunit Knockout Mice

The role of the δ subunit is poorly defined. At the electrophysiological level, in hippocampal slices from δ knockout mice, a significantly faster miniature inhibitory postsynaptic current decay time was found, with no change in miniature inhibitory postsynaptic current amplitude or frequency (70). However, the neurosteroid THDOC (3α, 21-dihydroxy-5α-pregnan-20-one) failed to prolong IPSCs in cerebellar granule neurons (71), suggesting a role for δ-containing receptors in neurosteroid action. In neurons in the ventral basal complex of the thalamus from the δ knockout mice, pregnenolone sulfate reduced evoked IPSC amplitude and duration, in contrast to wild type, whereas spontaneous IPSCs were unchanged (72). These findings suggest that δ-containing $GABA_A$ receptors may be functionally limited to extrasynpatic sites.

In δ subunit knockout mice, the duration of the loss of the righting reflex was significantly decreased in response to the neurosteroids alphaxalone and pregnanolone, but not in response to pentobarbital, propofol, midazolam, etomidate, and ketamine (70). In the elevated plus maze, the neurosteroid ganaxalone had no anxiolytic-like effect in δ knockout mice, in contrast to wild-type mice (70). Likewise, ganaxalone failed to prolong pentylenetetrazole-induced absence-like immobilization in δ knockout mice, indicating that $GABA_A$ receptors containing the δ subunit are involved in mediating the anxiolytic-like and proabsence effects

of neurosteroids (70). Similarly, δ knockout mice develop spontaneous seizures and are more susceptible to pentylenetetrazole-induced convulsive seizures (73). In contrast to the results obtained with alphaxalone, the duration of the loss of the righting reflex in response to halothane was unaltered, as were the tail-clamp/withdrawal responses to halothane and enflurane (70). Contextual and tone fear conditioning were indistinguishable from wild type in the δ knockout, indicating no defect in these learning and memory functions (70). Moreover, δ knockout mice display reduced consumption of ethanol, attenuated withdrawal from chronic ethanol exposure, and a reduced anticonvulsant effect of ethanol, whereas the anxiolytic-like and hypothermic responses to ethanol and the development of acute and chronic tolerance were indistinguishable from wild type (70).

ρ1 Subunit Knockout Mice

The ionotropic GABA receptors consisting of ρ subunits display different properties than the receptor subtypes described above, for example, insensitivity to bicucculin. They are frequently named $GABA_C$ receptors (74), although in the official IUPHAR nomenclature, these receptors are included among the $GABA_A$ receptors (75). In the retina, the expression of these receptors is largely restricted to the terminals of retinal bipolar cells. These terminals transmit the visual signal to amacrine and ganglion cells. When the ρ1 subunit was genetically ablated, the expression of $GABA_C$ receptors was eliminated. Electroretinogram (ERG) analysis revealed that only inner retinal function was altered, indicating an alteration of the excitation/inhibition balance between second- and third-order retinal neurons (76).

DISSECTION OF BENZODIAZEPINE ACTION IN $GABA_A$ RECEPTOR POINT-MUTATED KNOCKIN MICE

As described above, studies with knockout mice have provided important insights into the function of $GABA_A$ receptor subtypes. However, adaptive compensatory changes during development and a high complexity or even lethality of the phenotype can make interpretation of findings difficult. In other words, it is frequently impossible to say whether an effect is missing because the knocked-out subunit is not expressed in the adult animal or whether the phenotype results from the lack of the subunit during development. Thus, the value of knockout animals in the delineation of the function of drug targets in adult animals has to be viewed with these restrictions in mind. Therefore, in a second wave of $GABA_A$ receptor-targeted mice, more subtle mutations, in particular, point mutations, were introduced into the mouse genome. The idea is that the point mutation would leave the physiological function of the receptor subunit and the receptor complex intact, thus largely avoiding developmental compensatory mechanisms. The point mutation would, however, specifically prevent the modulation of the respective receptor by defined

drugs. The first example chosen was a mutation in the benzodiazepine-binding site on the α subunit class. α Subunits containing a histidine residue at a conserved position (α1-H101, α2-H101, α3-H126, and α5-H105) can bind diazepam, whereas α subunits containing an arginine (α4-R99 and α6-R100) are unable to bind diazepam. When the histidine residue in the α1 subunit was replaced by an arginine residue, recombinant $\alpha 1\beta 2\gamma 2$ receptors became diazepam insensitive (77). Similar observations were made on receptors containing the α2, α3, and α5 subunits (78). Thus, for all four diazepam-sensitive α subunits, the exchange of a histidine to an arginine residue represents a genetic switch capable of rendering a specific $GABA_A$ receptor subtype diazepam insensitive.

Diazepam is used clinically for its anxiolytic, sedative, hypnotic, anticonvulsant, and myorelaxant actions. To explore the pharmacological role of defined $GABA_A$ receptor subtypes, α1(H101R), α2(H101R), α3(H126R), and α5(H105R) mice were generated (47, 49, 79, 80). The mutant mouse lines are expected to lack the diazepam effects normally mediated by the respective mutated $GABA_A$ receptor subtype. Any diazepam effect present in a particular mouse line must be mediated by one or more of the available wild-type receptor subtypes. Analysis by immunoblotting, immunohistochemistry, and immunofluorescence reveales that the mutant receptor subunits are expressed at normal levels, with unaltered regional and subcellular distributions. The notable exception is the α5(H105R) line, where the abundance of the (extrasynaptic) α5 subunit is reduced in the hippocampus (49). The regional and subcellular localization was, however, unchanged. The significance of this finding is discussed below.

α1(H101R) Knockin Mice

Whereas in wild-type mice diazepam decreases the horizontal motor activity, this effect was absent in α1(H101R) mice, demonstrating that in wild-type mice, the α1-containing $GABA_A$ receptors mediate the sedative action of diazepam (47). The neurosteroid 3α-hydroxy-5β-pregnan-20-one decreased motor activity in wild-type and α1(H101R) mice to the same extent, demonstrating that the lack of drug-induced sedation is restricted to benzodiazepine site ligands (47). In the passive avoidance paradigm, which provides a model for anterograde amnesia, diazepam decreases the latency to enter the dark compartment 24 h after receiving a foot shock in this compartment. This is indicative of the anterograde amnesic action of diazepam. In α1(H101R) mice, diazepam had no effect on this latency, indicating that the anterograde amnesic action of diazepam is also mediated by α1-containing $GABA_A$ receptors (47). The muscarinic antagonist scopolamine had an amnesic action in both wild-type and α1(H101R) mice, underscoring the specificity of α1-containing $GABA_A$ receptors in mediating diazepam-induced amnesia (47). Diazepam protects wild-type mice from pentylenetetrazole-induced myoclonic jerks and tonic seizures, whereas α1(H101R) mice are only partially protected (47). This suggests that the anticonvulsant activity of diazepam is mediated in part by α1-containing $GABA_A$ receptors. In contrast, the anticonvulsant activity

of sodium phenobarbital was indistinguishable in wild-type and α1(H101R) mice (47).

Whereas diazepam and other benzodiazepines have a high affinity for α1-, α2-, α3-, and α5-containing $GABA_A$ receptors, the imidazopyridine zolpidem, which is a widely used hypnotic, has a high affinity for α1-containing $GABA_A$ receptors, an approximately 20-fold-lower affinity at α2- and α3-containing $GABA_A$ receptors, and no affinity at α5-containing $GABA_A$ receptors, and can thus be considered an α1-selective agent. It decreased horizontal motor activity in wild-type but not α1(H101R) mice, indicating that α1-containing $GABA_A$ receptors also mediate the sedative action of zolpidem (81). In contrast to diazepam, zolpidem protected wild-type mice from tonic convulsions but not from myoclonic jerks. Its activity against myoclonic jerks was completely absent in α1(H101R) mice, indicating that this effect is exclusively mediated by α1-containing $GABA_A$ receptors (81).

The benzodiazepine site inverse agonists Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepine-3-carboxylate), a benzodiazepine, and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), a β-carboline, have effects that are opposite to those of classical benzodiazepine site agonists such as diazepam: They decrease the GABA-induced chloride currents. In wild-type mice, Ro15-4513 increases locomotor activity, whereas DMCM induces myoclonic jerks in 83% of wild-type mice (82). In a recombinant system, Ro15-4513 and DMCM act as agonists at α1(H101R)-containing $GABA_A$ receptors (78). In α1(H101R) mice, Ro15-4513 decreased locomotor activity and DMCM failed to elicit convulsions (82). These results demonstrate the contribution of α1-containing $GABA_A$ receptors to the pharmacological actions of benzodiazepine site inverse agonists.

When α1(H101R) mice are tested in a novel environment, diazepam increases the locomotor activity, an effect that is likely to be mediated by α2-, α3-, or α5-containing $GABA_A$ receptors (79, 83). In contrast, in a familiar environment, diazepam decreases motor activity in wild-type but not in α1(H101R) mice (47). Thus, the novelty or familiarity of the test environment is a parameter that critically affects the motor behavior.

In α1(H101R) mice, diazepam retained its anxiolytic-like activity in the light/dark choice test and the elevated plus maze test, its myorelaxant activity in the horizontal wire test, and its ethanol-potentiating effect as measured by the loss of the righting reflex (47), indicating that these activities are mediated by $GABA_A$ receptors containing the α2, α3, or α5 subunits.

When α1(H101R) mice are treated with diazepam, diazepam displays agonist activity at α2-, α3-, and α5-containing $GABA_A$ receptors. A new ligand, L-838,417, has been described that is a partial agonist at α2-, α3-, and α5-containing $GABA_A$ receptors but has no agonist activity at α1-containing $GABA_A$ receptors (79). This ligand would thus be predicted to have actions in wild-type mice that are similar to diazepam's actions in α1(H101R) mice. Indeed, in rats, L-838,417 displayed anxiolytic-like activity in the elevated plus maze and the fear-potentiated

startle, whereas it did not impair the motor performance of wild-type mice on the rotarod (79).

Because the sedative action of diazepam is mediated by $GABA_A$ receptors containing the $\alpha 1$ subunit, it was expected that the hypnotic activity of diazepam would also be mediated by this receptor subtype. However, in $\alpha 1$(H101R) mice, 3 mg/kg diazepam reduced the amount of initial REM sleep similar to wild-type mice. Furthermore, the increase in power density above 21 Hz in non-REM sleep and waking and the suppression of slow-wave activity (0.75 Hz–4 Hz) in non-REM sleep were seen in both $\alpha 1$(H101R) and wild-type mice, being even more pronounced in the $\alpha 1$(H101R) mice. In addition, the number of brief awakenings per hour of sleep was decreased and thus sleep continuity was enhanced by diazepam only in $\alpha 1$(H101R) mice (84). These findings suggest that the observed diazepam-induced changes in the EEG and possibly its hypnotic action are not mediated by $\alpha 1$-containing $GABA_A$ receptors. This indicates that the sedative and EEG/hypnotic actions of diazepam are qualitatively, and not only quantitatively, distinct phenomena mediated by different neuronal circuits.

$\alpha 2$(H10R) Knockin Mice

$GABA_A$ receptors in the limbic system and in the reticular activating system have been postulated to mediate the therapeutically most important anxiolytic action of benzodiazepines. $\alpha 2$-Containing $GABA_A$ receptors are expressed in the limbic system in regions that are involved in emotional stimulus processing (85), e.g., amygdala and hippocampus. In the light-dark choice test and the elevated plus maze test, diazepam increased the time the mice spent in the lit compartment or the open arms, respectively, in wild-type mice, but not in $\alpha 2$(H101R) mice (80). This indicates that $\alpha 2$-containing $GABA_A$ receptors, which constitute approximately 15% of the diazepam-sensitive $GABA_A$ receptors (86), mediate the anxiolytic-like action of diazepam. Because diazepam still acts on receptor subtypes containing the $\alpha 1$, $\alpha 3$, and $\alpha 5$ subunits in $\alpha 2$(H101R) mice, the experiments do not necessarily predict whether, e.g., an $\alpha 3$-selective agonist may also display an anxiolytic-like action in the light/dark choice test or the elevated plus maze test.

$\alpha 2$(H101R) mice displayed normal sedative and anticonvulsant responses to diazepam (80); however, the myorelaxant action as measured in the horizontal wire test was strongly impaired (87). Appreciably higher doses of diazepam were required to induce myorelaxation compared to its anxiolytic-like effect (80). The myorelaxant action of the $GABA_B$ receptor agonist baclofen was indistinguishable in $\alpha 2$(H101R) and wild-type mice (87).

$\alpha 3$(H126R) Knockin Mice

The $GABA_A$ receptor $\alpha 3$ subunit is expressed, e.g., in monoaminergic and serotonergic neurons of the brain stem and in basal forebrain cholinergic neurons. In the light/dark choice test and the elevated plus maze test, the anxiolytic-like action of diazepam is retained in $\alpha 3$(H126R) mice, indicating that $\alpha 3$-containing

receptors are not required for the anxiolytic-like action of diazepam in these tests. It is interesting to note, however, that an apparently $\alpha 3$-selective inverse agonist, compound 8g, is reported to be anxiogenic in the rat elevated plus maze test (88). Furthermore, the sedative and anticonvulsant activities of diazepam were unchanged (80). However, the muscle relaxant activity was moderately reduced, indicating that $\alpha 3$-containing receptors mediate this response in part (87). Despite $\alpha 3$ being the exclusive α subunit in the reticular nucleus of the thalamus, $\alpha 3$-containing $GABA_A$ receptors do not mediate diazepam's effect on the sleep EEG because the diazepam-induced EEG changes did not differ between $\alpha 3$(H126R) and wild-type mice (89). The reticular nucleus of the thalamus regulates thalamocortical oscillations. The suppression of thalamic oscillations by clonazepam is retained in slices of $\alpha 1$(H101R) mice but not $\alpha 3$(H126R) mutant mice, indicating that this suppression is mediated exclusively by $\alpha 3$-containing $GABA_A$ receptors (90). Thus, $\alpha 3$-containing $GABA_A$ receptors are an interesting target for novel anti-absence drugs.

$\alpha 5$(H105R) Knockin Mice

In $\alpha 5$(H105R) mice, the sedative, anticonvulsant, and anxiolytic-like action of diazepam were indistinguishable from wild type. Only the muscle relaxant action of diazepam was reduced in these mutant mice (49). Thus, the myorelaxant action of diazeapam appears to be mediated largely by $\alpha 2$-, $\alpha 3$-, and $\alpha 5$-containing $GABA_A$ receptors. At a moderate dose of diazepam (e.g., at 10 mg/kg), the role of the $\alpha 2$-containing $GABA_A$ receptors appears to be essential because $\alpha 2$(H101R) mice do not display muscle relaxation at this dose in contrast to $\alpha 3$(H126R) and $\alpha 5$(H105R) mice (49, 87).

Whereas in $\alpha 1$(H101R), $\alpha 2$(H101R), and $\alpha 3$(H126R) mice the mutant subunits have been found to be expressed at normal levels, the expression of the mutant $\alpha 5$ subunits in $\alpha 5$(H105R) mice was reduced in hippocampal pyramidal cells by approximately 30%, but not in other brain regions. However, the laminar distribution of the remaining $\alpha 5$ subunit in the hippocampus was unchanged (49). The reasons for this selective reduction are not known. In contrast to the $\alpha 1$, $\alpha 2$, and $\alpha 3$ subunits, the $\alpha 5$ subunit in the hippocampus is located largely extrasynaptically.

In line with the role of the hippocampus in certain forms of associative learning, $\alpha 5$(H105R) mice displayed selective changes in learning and memory performance. When a tone (conditioned stimulus) and a foot shock (unconditioned stimulus) are paired, associative learning involves the hippocampus, provided tone and foot shock are separated by a time interval (trace fear conditioning) but not when they coterminate or overlap (delay fear conditioning). When the $\alpha 5$(H105R) mice were tested in a delay fear conditioning paradigm, the amount of freezing was the same as in wild-type mice. However, in trace fear conditioning, the $\alpha 5$(H105R) mice showed an enhanced percentage of freezing compared to wild-type mice (49). These findings implicate the extrasynaptic hippocampal $\alpha 5$-containing $GABA_A$ receptors as control elements of the temporal association between the two cues in

trace fear conditioning. In addition, as mentioned in $\alpha 5$ Subunit Knockout Mice, above, $\alpha 5$ subunit knockout mice showed an improved performance in a particular Morris water maze test (32).

Interestingly, the time spent freezing to the tone in trace fear conditioning was also increased in the $\gamma 2+/-$ mice heterozygous for the $\gamma 2$ null mutation (see also $\gamma 2$ Subunit Knockout Mice, above), which display a loss of benzodiazepine-binding sites in the CA1 and CA3 regions of 35% and 28%, respectively (65). These studies demonstrate that, most likely, $GABA_A$ receptors containing the $\alpha 5$ subunit and the $\gamma 2$ subunit are crucial for this response. There is also evidence for the involvement of excitatory neurotransmitter receptors in trace fear conditioning. Mice lacking the NMDA (N-methyl-D-aspartate) receptor subunit NR1 in CA1 pyramidal cells only (NR1.CA1-KO mutant) failed to memorize the tone-shock association in fear conditioning when tone and shock are temporally separated by a trace, but not in delay fear conditioning, when the trace was removed (91). Thus, hippocampal NMDA receptors and $GABA_A$ receptors containing the $\alpha 5$ subunit and/or the $\gamma 2$ subunit are able to modulate the freezing response in trace fear conditioning in opposite directions.

DISSECTION OF GENERAL ANESTHETIC ACTION IN $GABA_A$ RECEPTOR POINT-MUTATED KNOCKIN MICE

General anesthetics were introduced into clinical practice more than 150 years ago; however, their mechanism of action is still not completely understood. The observations that a wide range of chemicals can induce anesthesia, and that their anesthetic potency appears to be directly related to their lipid solubility, has led to the hypothesis that they may unspecifically perturb the lipid bilayer. Later, it was discovered that general anesthetics modulate the activity of neuronal ion channels, e.g., $GABA_A$ receptors, NMDA receptors, 5-HT3 receptors, and two-pore domain potasssium channels in vitro (92, 93). However, it is not clear which neuronal target would mediate individual actions of general anesthetics in vivo.

$\beta 3$(N265M) Mice

The expression of the $\beta 3$ subunit overlaps that of the $\beta 2$ subunit in cerebral cortex, cerebellum, and most layers of the olfactory bulb, and high expression of $\beta 3$ is observed in the corpus striatum and in the granule cells of the olfactory bulb. In the hippocampus, $\beta 3$ expression is higher than that of $\beta 2$. $\beta 2$, but not $\beta 3$, is expressed in thalamic nuclei, substantia nigra, globus pallidus, inferior colliculus, and the short axon cells of the olfactory bulb (94). Furthermore, $\beta 3$ is strongly expressed in the spinal cord (95). Point mutations in α and β subunits of recombinant $GABA_A$ receptors have been described that abolish the modulatory and/or direct actions of some general anesthetics (96, 97). One of these point mutations, N265M, was introduced into the $\beta 3$ subunit gene of the mouse to

generate β3(N265M) mice. This point mutation essentially abolishes the modulatory and direct effects of the intravenous anesthetics etomidate and propofol and substantially reduces the modulatory actions of the volatile anesthetic enflurane. In contrast, the modulatory action of the neurosteroidal intravenous anesthetic alphaxalone is preserved (98). In hippocampal pyramidal neurons of β3(N265M) mice, the potentiation of GABA-induced chloride currents by etomidate was substantially reduced. Because β3 is the predominant, but not exclusive, β subunit in these neurons, this result indicates that β3-containing neurons have become etomidate-insensitive (99). In the β3(N265M) mice, etomidate and enflurane were significantly less effective in decreasing spontaneous action potential firing in cultured cortical brain slices (99). To assess the hypnotic and immobilizing actions of etomidate and propofol in β3(N265M) mice, the loss of the righting reflex and the loss of the hindlimb withdrawal reflex were examined. Both anesthetics lead to a strong decrease in the duration of the loss of the righting reflex. Even more strikingly, the hindlimb withdrawal reflex was essentially completely absent in the β3(N265M) mice, indicating that the immobilizing effect of etomidate and propofol is critically dependent on β3-containinig $GABA_A$ receptors (99). We speculate that β3-containing $GABA_A$ receptors in the spinal cord mediate this effect to a large degree because previous research suggested that the immobilizing action of isoflurane and propofol is mediated at the spinal cord level (100, 101). The actions of the neurosteroidal mix alphaxalone/alphadolone were indistinguishable in β3(N265M) and wild-type mice, indicating that the β3-containing $GABA_A$ receptors in the β3(N265M) mice are functional (99). The concentrations of the volatile anesthetics enflurane and halothane needed for a loss of the righting reflex were identical in β3(N265M) and wild-type mice, indicating that β3-containing $GABA_A$ receptors are not mediating this response (99). The β3(N265M) mice were, however, less sensitive to the immobilizing action of enflurane and halothane, indicating that β3-containing $GABA_A$ receptors are partly mediating this effect. Notably, at higher concentrations of enflurane and halothane, all mice lost the hindlimb withdrawal reflex, indicating that at higher concentrations other targets are sufficient for the immobilizing action (99). Essentially identical results for enflurane and halothane have also been obtained when studying β3 knockout mice (59) (see also β3 Subunit Knockout Mice, above). Thus, in contrast to α1, where the α1(H101R) and the knockout allele provide opposite results with respect to the sedative action of diazepam, the β3(N265M) and the knockout allele display the same phenotype with respect to enflurane and halothane action. Although the duration of the loss of the righting reflex in response to etomidate is reduced in β3 knockout mice compared to wild type (59), the loss of the hindlimb withdrawal reflex or tail-clamp/withdrawal reflex in response to etomidate or propofol has not been studied in the β3 knockout mice. Taken together, the results show that a single molecular target is a major determinant of behavioral responses evoked by the intravenous anesthetics etomidate and propofol, whereas the volatile anesthetics appear to act via a broader spectrum of targets.

OUTLOOK

Studies on $GABA_A$ receptor subunit knockout and knockin mice have provided considerable insights into the physiological, pharmacological, and behavioral role of individual $GABA_A$ receptor subtypes. In the future, it is expected that temporally and/or spatially controlled deletion of genes will help to refine the precision of analysis. Drugs that specifically target defined receptor subtypes are currently under development and are expected to provide novel therapeutic opportunities.

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