Inhibitory Ligand-Gated Ion Channels as Substrates for General Anesthetic Actions

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Abstract General anesthetics have been in clinical use for more than 160 years. Nevertheless, their mechanism of action is still only poorly understood. In this review, we describe studies suggesting that inhibitory ligand-gated ion channels are potential targets for general anesthetics in vitro and describe how the involvement of γ-aminobutyric acid $(GABA)_A$ receptor subtypes in anesthetic actions could be demonstrated by genetic studies in vivo.

1 Introduction

In 1846 the first public demonstration of anesthesia with ether by William T. Morton at the Massachusetts General Hospital in Boston heralded a new era in medical practice, in particular enabling the performance of sophisticated surgical operations that would not be possible without general anesthesia. It was soon discovered that a variety of substances have general anesthetic actions. About a century ago, Meyer

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and Overton independently discovered a strong correlation between anesthetic potency and solubility in oil (Meyer-Overton rule). These observations led to the view that general anesthetics act in the lipid bilayer of the neuronal plasma membrane by an unspecific mechanism (lipid theory). However, Franks and Lieb demonstrated that general anesthetics can interact directly with proteins (protein theory), and that the interaction with proteins also fulfills the predictions of the Meyer-Overton rule (Franks and Lieb 1984). The fact that optical isomers of some anesthetics differ in potency also cannot be explained by a nonspecific action (Franks and Lieb 1994). Moreover, substances have been identified that would be predicted by the Meyer-Overton rule to be anesthetic, but they are in fact not ("non-immobilizers"), and the "long chain alcohol cutoff," i.e., the observation that alcohols that exceed a certain size are inactive, also cast doubt on the lipid theory (Koblin et al. 1994). Today there is ample evidence that anesthetics directly modulate ion channels. These interactions can be both specific and unspecific in nature (Urban et al. 2006).

Over time it became apparent that general anesthetics modulate the activity of ion channels in the membrane of nerve cells at clinically relevant concentrations (Krasowski and Harrison 1999; Yamakura and Harris 2000). With respect to the inhibitory ligand-gated ion channels, it is noteworthy that etomidate, propofol, barbiturates, isoflurane, and sevoflurane significantly increase the activity of γ-aminobutyric acid $(GABA)_A$ receptors at clinically relevant concentrations, while ketamine and nitrous oxide apparently do not modulate the activity of $GABA_A$ receptors to a significant degree at these concentrations. At the glycine receptor, isoflurane and sevoflurane significantly increase glycine-induced chloride currents at clinically relevant concentrations, while propofol, etomidate, barbiturates, and nitrous oxide display smaller effects (Belelli et al. 1999). Ketamine does not modulate the glycine receptor (Krasowski and Harrison 1999). However, one should note that the observation that a certain general anesthetic modulates a specific class of ligand-gated ion channels or a subtype thereof in vitro does not tell us whether this ion channel subtype is responsible for mediating any of the effects of this general anesthetic in vivo. Another caveat is that recombinant systems may not contain receptor-associated proteins that may influence anesthetic sensitivity of a particular receptor.

2 Inhibitory Ligand-Gated Ion Channels: $GABA_A$ and Glycine Receptors

$GABA_A$ receptors are involved in the regulation of vigilance, anxiety, memory, and muscle tension. They are pentameric complexes with six α-, three β-, one δ-, one ε-, one π-, one θ-, and three ρ-subunit genes known. Most $GABA_A$ receptors appear to consist of α-, β-, and γ-subunits, believed to be assembled in a 2:2:1 stoichiometry. Preferred combinations include $\alpha_1\beta_2\gamma_2$ (representing ca. 60% of all $GABA_A$ receptors in the brain), $\alpha_2\beta_3\gamma_2$ (15%), and $\alpha_3\beta_n\gamma_2$ (10%–15%). The subunit combinations $\alpha_4\beta_2\gamma$, $\alpha_4\beta_n\delta$, $\alpha_5\beta_{1/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$, and $\alpha_6\beta_n\delta$ each represent less than 5% of all receptors in the brain (McKernan and Whiting 1996; Mohler et al. 2002). $GABA_A$ receptors can be found in both synaptic and extrasynaptic locations.

For practical purposes, $GABA_A$ receptors are frequently classified on the basis of their α- and β-subunits as α_n-containing $GABA_A$ receptors and β_n-containing $GABA_A$ receptors, respectively.

Glycine receptors also belong to the family of ligand-gated ion channels. They appear to be particularly prevalent in the brain stem and spinal cord. There are four α-subunits and a single β-subunit known, with receptors comprising α-homomers or αβ-heteromers. Most glycine receptors in adult animals are of the $\alpha_1\beta$ type. Volatile anesthetics such as halothane, isoflurane, and sevoflurane strongly potentiate the glycine-induced chloride currents at clinically relevant concentrations in recombinant systems and also in neurons (Harrison et al. 1993; Downie et al. 1996; Mascia et al. 1996; Krasowski and Harrison 1999), while the potentiation by propofol at clinically relevant concentrations is much smaller, suggesting that if glycine receptors play a significant role in clinical anesthesia, this would likely be restricted to volatile anesthetics (Belelli et al. 1999; Grasshoff and Antkowiak 2004). The enflurane- or isoflurane-induced depression of spontaneous action potential firing in ventral horn interneurons in spinal cord cultures has recently been found to be mediated almost equally by $GABA_A$ receptors and glycine receptors (Grasshoff and Antkowiak 2006). Clearcut in vivo data demonstrating that glycine receptors would mediate specific anesthetic actions are currently unavailable.

As pointed out previously, it has been known for some time that most general anesthetics modulate the activity of $GABA_A$ receptors in vivo at clinically relevant concentrations (Krasowski and Harrison 1999). In vitro studies suggest that ketamine and nitrous oxide do not act via $GABA_A$ receptors (Krasowski and Harrison 1999). $GABA_A$ receptor agonistic actions of ketamine have been proposed based on pharmacological in vivo data (Irifune et al. 2000), but other in vivo studies reported that the $GABA_A$ antagonist gabazine did not block ketamine-induced anesthesia (Nelson et al. 2002; Sonner et al. 2003). It has also been reported that nitrous oxide, tested at a concentration (100%, 29.2 mM) that is higher than that used clinically, increases the efficacy of GABA at recombinant $GABA_A$ receptors (Hapfelmeier et al. 2000). At higher concentrations, some general anesthetics also directly activate the $GABA_A$ receptor in the absence of GABA; the pharmacological relevance of this observation is currently unknown. Since most general anesthetics modulate the activity of a variety of neuronal ion channels, in particular ligand-gated ion channels, it is impossible to draw conclusions from in vitro data as to which neuronal ion channels (or other neuronal targets) mediate clinically relevant actions of general anesthetics.

3 Targeted Mutations in $GABA_A$ Receptor Subunit Genes

3.1 $GABA_A$ Receptor Subunit Knockout Mice

Knockout mice with deletions of specific $GABA_A$ receptor subunits potentially provide a valuable tool for assessing physiological or pharmacological functions of the respective $GABA_A$ receptor subunits. For various reasons this approach has met

with variable success. Potential problems include compensatory mechanisms, e.g., upregulation of related subunits, and influence on the expression of neighboring genes due to enhancers in the neomycin expression cassette. This is especially problematic for $GABA_A$ receptor subunits since the genes are arranged in clusters (Uusi-Oukari et al. 2000) and multiple impairments may make it difficult to distinguish primary and secondary effects of a knockout. In mice with a knockout of the β_3 subunit (Homanics et al. 1997) the duration of the loss of the righting reflex in response to midazolam and etomidate–but not to pentobarbital, enflurane, halothane, and ethanol–was reduced compared to wildtype mice, and the immobilizing action of halothane and enflurane, as determined in the tail clamp withdrawal test, was decreased (Quinlan et al. 1998). These results point to a role of β_3-containing $GABA_A$ receptors in the hypnotic and immobilizing actions of the drugs mentioned, but it is also worth noting that when the enflurane-induced depression of spinal cord neurotransmission was examined in spinal cord slices of these mice, it was found that other targets substitute for the role that is normally played by β_3-containing $GABA_A$ receptors (Wong et al. 2001).

In δ-subunit knockout mice, the duration of the loss of the righting reflex was significantly decreased in response to the neuroactive steroid alphaxalone and the neurosteroid pregnenolone, but not in response to midazolam, etomidate, propofol, pentobarbital, and ketamine, indicating the potential involvement of δ-containing $GABA_A$ receptors in the actions of neurosteroidal anesthetics (Mihalek et al. 1999).

Another mouse model that has provided valuable information on targets mediating actions of general anesthetics is the α_5 knockout mouse (Collinson et al. 2002). In α_5 knockout mice, the duration of the loss of the righting reflex in response to etomidate was indistinguishable from wildtype mice, indicating that α_5-containing $GABA_A$ receptors do not mediate the hypnotic action of etomidate (Cheng et al. 2006). It was, however, found that the amnestic action of etomidate in a contextual fear conditioning paradigm and in the Morris water maze (a test for hippocampal learning) are absent in α_5 knockout mice, indicating that these actions of etomidate are mediated by α_5-containing $GABA_A$ receptors (Cheng et al. 2006).

3.2 $GABA_A$ Receptor Subunit Knockin Mice

In an attempt to circumvent some of the problems encountered when studying knockout mice, knockin mice carrying point mutations were generated. These point mutations were designed to alter the sensitivity of the respective receptor subtype to CNS-depressant drugs, while largely maintaining the sensitivity for the physiological neurotransmitter GABA. Even if the mutations are not completely "silent," knockin mice offer substantial insights into the functions of defined $GABA_A$ receptors in the actions of general anesthetics (Rudolph and Mohler 2004).

A conserved histidine residue in the extracellular N-terminal domain of α_1, α_2, α_3, and α_5 subunits is required for binding of classical benzodiazepines like

diazepam (Wieland et al. 1992; Kleingoor et al. 1993; Benson et al. 1998). In mice with the $\alpha_1(H101R)$ mutation in the α_1 subunit, diazepam does not reduce motor activity, indicating that the sedative action of diazepam is mediated by α_1-containing $GABA_A$ receptors (Rudolph et al. 1999; Crestani et al. 2000; McKernan et al. 2000). It is noteworthy that in α_1 knockout mice diazepam still decreases locomotor activity, even more strongly than in wildtype mice (Kralic et al. 2002b; Reynolds et al. 2003a), so that studies in knockout and knockin mice would apparently lead to opposing conclusions. Interestingly, L-838,417, a benzodiazepine site ligand that is an antagonist at α_1-containing $GABA_A$ receptors but a partial agonist at α_2-, α_3-, and α_5-containing $GABA_A$ receptors, also has no sedative action (McKernan et al. 2000), confirming the conclusion obtained with the $\alpha_1(H101R)$ knockin mice by two independent groups and suggesting that the strong upregulation of the α_2 and α_3 subunits in the α_1 knockout mice (Sur et al. 2001; Kralic et al. 2002a) makes these mice sensitive to diazepam-induced sedation. Furthermore, α_1 knockout mice have been found to display an increased tonic $GABA_A$ receptor-mediated current in cerebellar granule cells, which is likely due to a reduction of GABA transporter (GAT) activity, which thus might represent another adaptive mechanism (Ortinski et al. 2006). Studies with $\alpha_1(H101R)$ knockin mice also suggest that α_1-containing $GABA_A$ receptors mediate the anterograde amnesic action and in part the anticonvulsant actions of diazepam (Rudolph et al. 1999). The anxiolytic-like action of diazepam is absent in $\alpha_2(H101R)$ mice, indicating that sedation and anxiolysis are mediated by distinct receptor subtypes and can be separated pharmacologically (Low et al. 2000). The myorelaxant action of diazepam, determined in the horizontal wire test, is mediated primarily by α_2-, but also by α_3- and α_5-containing $GABA_A$ receptors (Crestani et al. 2001, 2002).

In pioneering studies using recombinant receptors, amino acid residues in the second and third transmembrane domain of α- and β-subunits have been identified that are crucial for the action of many general anesthetic agents on $GABA_A$ receptors. Sites on both α- and β-subunits have been found to be involved in the action of volatile anesthetics such as enflurane and isoflurane. These include (but are not limited to) α_1-S270, α_1-A291, $\beta_{2/3}$-N265, and $\beta_{2/3}$-M286 (Belelli et al. 1997; Mihic et al. 1997; Krasowski et al. 1998; Siegwart et al. 2002, 2003). In contrast, only sites on the β-subunits have been found to be relevant for the actions of the intravenous anesthetics etomidate and propofol (Belelli et al. 1997; Krasowski et al. 1998). The replacement of an asparagine in position 265 of β_2 or β_3 with methionine [the residue found in the homologous position of the *Drosophila melanogaster Rdl* $GABA_A$ receptor, which is insensitive to etomidate (Pistis et al. 1999)] results in a profound decrease of the modulatory and direct (i.e., GABA-independent) actions of etomidate and propofol (Belelli et al. 1997; Siegwart et al. 2002, 2003). The potency of etomidate is roughly ten times smaller at β_1- compared to β_2- and β_3-containing $GABA_A$ receptors (Hill-Venning et al. 1997). The β_1 subunit contains a serine residue at position 265 that is responsible for this property (Belelli et al. 1997; Hill-Venning et al. 1997). Although the β_2- and β_3-containing $GABA_A$ receptors appear to be the prime targets for etomidate, it cannot be formally excluded that β_1-containing $GABA_A$ receptors still

may contribute to the clinical actions of etomidate. Moreover, multiple known [e.g., 11β-hydroxylase, α_2B and α_2C adrenoceptors (Paris et al. 2003)] and potentially also unknown targets for etomidate exist. If a mutation e.g., in the $GABA_A$ receptor β_2 subunit renders the respective $GABA_A$ receptor subtype insensitive to etomidate, one should be careful with the conclusion that any remaining etomidate action is mediated by β_3-containing $GABA_A$ receptors, although this is not unlikely. Furthermore it has been shown recently that $GABA_A$ receptor subtypes containing β_1 and rare subunits such as θ may be sensitive to etomidate. Specifically, recombinant $\alpha_3\beta_1\theta$ $GABA_A$ receptors have a higher efficacy for etomidate compared to $\alpha_3\beta_1$ or $\alpha_3\beta_1\gamma_2$ receptors, although the potency for etomidate was apparently unchanged (Ranna et al. 2006).

4 Studies of General Anesthetic Actions In Vivo

4.1 Intravenous Anesthetics: Etomidate and Propofol

4.1.1 Immobilization and Hypnosis

The first knockin mouse model harboring a $GABA_A$ receptor insensitive to a clinically used general anesthetic was the *β_3(N265M)* knockin mouse (Jurd et al. 2003). In vitro, this point mutation completely abolished the modulatory and direct effects of etomidate and propofol and substantially reduced the modulatory action of enflurane. However, the modulatory action of the neuroactive steroid alphaxalone was preserved (Siegwart et al. 2002). In neocortical slices of *β_3(N265M)* knockin mice, etomidate and enflurane were less effective at decreasing spontaneous action potential firing (Jurd et al. 2003). In hippocampal CA1 pyramidal neurons, the modulatory action of etomidate was reduced, consistent with the β_3 subunit being the predominant, but not exclusive, β-subunit in these cells (Jurd et al. 2003). Motor activity and hot plate sensitivity were unchanged in the absence of drugs (Jurd et al. 2003).

As a measure of the immobilizing action of etomidate and propofol, the hindlimb withdrawal reflex, which is lost in response to these drugs, was studied. The absence of this reflex is indicative of surgical tolerance (Arras et al. 2001). In the *β_3(N265M)* knockin mice the loss of the hindlimb reflex in response to etomidate and propofol that is invariably seen in wildtype mice was absent, indicating that the immobilizing action of these agents is apparently completely dependent on β_3-containing $GABA_A$ receptors (Fig. 1; Jurd et al. 2003). To monitor the hypnotic action of etomidate and propofol, the righting reflex was studied. Etomidate and propofol abolished the righting reflex in wildtype mice. In the *β_3(N265M)* knockin mice the duration of the loss of the righting reflex in response to these drugs was significantly reduced, indicating that the hypnotic action of etomidate and propofol is mediated in part by β_3-containing $GABA_A$ receptors (Fig. 1; Jurd et al. 2003). This essential phenotype of the *β_3(N265M)*

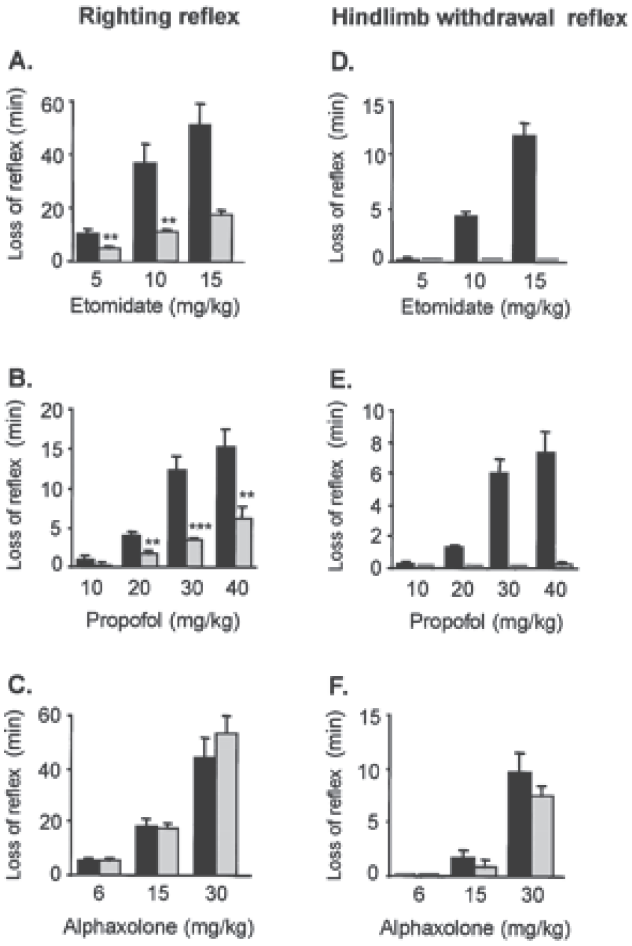

Fig. 1 Behavioral responses to i.v. anesthetics in wildtype and $\beta_3(N265M)$ mice. Reduction in the duration (in minutes) of the loss of righting reflex (LORR) induced by **a** etomidate and **b** propofol in $\beta_3(N265M)$ mice vs wildtype. Etomidate (15 mg/kg) and propofol (40 mg/kg) were lethal for 50% and 58% of the wildtype, respectively, but none of the $\beta_3(N265M)$ mice. **c** Alphaxalone [mixed in a 3:1 ratio with alphadolone, Saffan (Vet Drug, Dunnington, UK)] induced a similar duration (also given in minutes) of LORR in both genotypes. At 30 mg/kg, alphaxalone was lethal in 67% of wildtype mice and 50% of $\beta_3(N265M)$ mice. **d** Etomidate (10, 15 mg/kg) and **e** propofol (20, 30 mg/kg) failed to induce loss of the hind limb withdrawal reflex (LHWR) in $\beta_3(N265M)$ mice in contrast to wildtype mice ($p<0.01$, Fischer's exact test). **f** Alphaxalone (15, 30 mg/kg) induced LHWR with similar duration in $\beta_3(N265M)$ and wildtype mice. All drugs were administered intravenously. Wildtype mice, *black shading*, $\beta_3(N265M)$ mice, *gray shading*. $^{**}p<0.01$, $^{***}p<0.001$, compared with wildtype; median test (n=6–12 per group). (Reprinted with permission from *FASEB Journal*, Jurd et al. 2003)

knockin mice has now been observed on three different genetic backgrounds (129X1/SvJ×129/Sv (87.5%/12.5%) (Jurd et al. 2003), 129X1/SvJ (10 backcrosses), and C57BL/6J (9 backcrosses) (Zeller et al. 2007a), indicating that this

phenotype is very robust and also that *Gabrb*3, which is located between 57.4 and 57.7 Mb, is different from a gene that has been described as *lorp*1(loss or righting reflex in response to propofol), which has been mapped with a 99% confidence interval to 71.4–89.7 Mb on mouse chromosome 7 (Simpson et al. 1998); in addition, an etomidate-sensitivity quantitative trait locus (QTL) has also been identified in this chromosome region (Christensen et al. 1996; Downing et al. 2003). Thus, there is good evidence that the lack of immobility and partial lack of hypnosis in response to etomidate and propofol is really due to the N265M point mutation in the *Gabrb*3 gene.

In a parallel experiment performed by another group, the asparagine-265 residue in the β_2 subunit was replaced by a serine residue. A serine residue is found in the homologous position of the "etomidate-insensitive" β_1 subunit. This mutation abolishes the action of etomidate, but not of propofol. In cerebellar Purkinje cells of *β_2(N265S)* knockin mice, which predominantly contain $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, the modulatory effect of etomidate was substantially reduced (Reynolds et al. 2003b). The pedal withdrawal reflex in response to etomidate was still present in *β_2(N265S)* knockin mice, although its duration was reduced (Reynolds et al. 2003b). Injection of propofol led to a loss of the reflex in both wildtype and *β_2(N265S)* knockin mice, compatible with the point-mutated β_2-containing receptors being sensitive for propofol (Reynolds et al. 2003b). The duration of the loss of the righting reflex in response to etomidate was reduced in *β_2(N265S)* knockin mice compared to wildtype mice, whereas the response to propofol was identical in both genotypes, consistent with the mutant receptors being sensitive to propofol (Reynolds et al. 2003b).

The results of these studies with *β_3(N265M)* and *β_2(N265S)* knockin mice suggest that the immobilizing action of etomidate and propofol is mediated largely by β_3-containing $GABA_A$ receptors, whereas its hypnotic action is mediated by both β_2- and β_3-containing $GABA_A$ receptors. While the neurocircuitry responsible for the righting reflex are largely unknown, previous research has shown that the immobilizing actions of propofol are mediated at the spinal cord level (Antognini and Schwartz 1993; Rampil et al. 1993; Rampil 1994; Antognini et al. 2000). Thus, it is conceivable that β_3-containing $GABA_A$ receptors in the spinal cord play an important role in mediating the immobilizing action of etomidate and propofol.

Furthermore, the $GABA_A$ receptor antagonists gabazine systemic und picrotoxin increased the ED_{50} for propofol-induced immobilization in rats (Sonner et al. 2003), and the $GABA_A$ receptor antagonist bicuculline antagonized the hypnotic action of propofol (Irifune et al. 2003). While these studies provide strong evidence for an involvement of $GABA_A$ receptors in propofol-induced immobilization, they did not identify which $GABA_A$ receptor subtype would mediate this action. In another study, muscimol (an agonist of the $GABA_A$ receptor at the GABA site), propofol, and pentobarbital, administered intracerebroventricularly, led to a loss of the righting reflex [which these authors termed "sedation" but which in our terminology represents "hypnosis" (see also Rudolph and Antkowiak 2004)]. The actions of these drugs were attenuated by systemic gabazine (Nelson et al. 2002). All three agents were found to increase c-fos staining in the ventrolateral preoptic nucleus (VLPO)

and decrease c-fos-staining in the tuberomammillary nucleus (TMN), indicating that they increase neuronal activity in the VLPO and decrease neuronal activity in the TMN, which is an arousal-producing nucleus (Nelson et al. 2002). The VLPO is known to release GABA into the TMN, thus likely causing inhibition of the TMN, which releases histamine in the cortex. Direct injection of muscimol into the TMN results in a loss of the righting reflex, indicating that the action of muscimol in the TMN is sufficient for its hypnotic effect (Nelson et al. 2002). When propofol and gabazine are administered systemically, gabazine, administered into the TMN, reduced the duration of the loss of the righting reflex, indicating that the TMN plays a role in the hypnotic actions of propofol and pentobarbital (Nelson et al. 2002). Since VLPO and TMN are known to be a part of the non-rapid eye movement (REM) sleep-promoting pathway, this work provides an interesting potential connection between anesthesia and sleep.

4.1.2 Sedation

At subanesthetic doses, etomidate decreases motor activity, i.e., exerts a sedative action. This sedative action is observed in *β_3(N265M)* knockin mice (Zeller et al. 2005), but not in *β_2(N265S)* knockin mice (Reynolds et al. 2003b). These results suggest that the sedative action of etomidate is mediated by β_2-containing $GABA_A$ receptors but not by β_3-containing $GABA_A$ receptors. $\alpha_1\beta_2\gamma_2$ is the most abundant $GABA_A$ receptor subtype in the central nervous system (McKernan and Whiting 1996; Mohler et al. 2002). The observations that the sedative action of diazepam is mediated by α_1-containing $GABA_A$ receptors (Rudolph et al. 1999; McKernan et al. 2000) and that the sedative action of etomidate is mediated by β_2-containing $GABA_A$ receptors (Reynolds et al. 2003b) suggest that the $\alpha_1\beta_2\gamma_2$ receptor subtype is the relevant subtype mediating the sedative, i.e., motor depressing actions, of CNS-depressant drugs. It is currently unknown which circuits or neuronal populations are involved in these actions. The observation that general anesthetics reduce activity prominently in cortical networks at sedative concentrations suggests that the cortex might play a prominent role (Hentschke et al. 2005). Etomidate caused impairment of motor performance in a rotating rod test that is indistinguishable between α_5 knockout mice and wildtype mice, which suggests that α_5-containing $GABA_A$ receptors do not mediate the motor impairing action of etomidate in this assay (Cheng et al. 2006).

4.1.3 Hypothermia

At anesthetic doses, etomidate also has a strong hypothermic action. This action is strongly reduced in β_2(N265S) knockin mice (Cirone et al. 2004) and only slightly reduced in β_3(N265M) knockin mice (Zeller et al. 2005), indicating that it is largely mediated by β_2-containing $GABA_A$ receptors and only to a small degree by β_3-containing $GABA_A$ receptors.

4.1.4 Respiratory and Cardiac Depression

When studying the immobilizing actions of etomidate and propofol in *β_3(N265M)* knockin mice and wildtype mice, Jurd and collaborators noticed that high doses of these drugs (etomidate 15 mg/kg i.v., propofol 40 mg/kg i.v.) are lethal for approximately 50% of the wildtype mice but not for *β_3(N265M)* knockin mice. Interestingly, alphaxalone/alphadolone (30/10 mg/kg i.v.) were lethal for approximately 50% of both wildtype and *β_3(N265M)* knockin mice (Jurd et al. 2003). These results suggest that the potentially lethal response is mediated by β_3-containing $GABA_A$ receptors. We hypothesized that either the cardiac depressant action or the respiratory depressant action of these general anesthetics might be responsible for the lethality observed.

In wildtype mice, etomidate and propofol induce a significant decrease in the heart rate. This decrease is also present in *β_3(N265M)* knockin mice, indicating that targets other than β_3-containing $GABA_A$ receptors mediate this effect (Zeller et al. 2005). Heart rate and temperature were determined at the same time. It is possible that the reductions in temperature and heart rate are interrelated and not independent phenomena.

Respiratory depression was assessed by monitoring arterial blood gases (PaO_2, $PaCO_2$) and pH values in samples taken from the carotid artery. After application of etomidate or propofol, the PaO_2 was significantly higher in *β_3(N265M)* knockin mice and the $PaCO_2$ was significantly lower in *β_3(N265M)* knockin mice compared to wildtype mice (Fig. 2; Zeller et al. 2005). The pH values were significantly higher in *β_3(N265M)* knockin mice compared to wildtype mice (Zeller et al. 2005). In contrast, there was no genotype difference in these parameters after application of a mixture of the neurosteroid anesthetics alphaxalone and alphadolone, demonstrating that *β_3(N265M)* knockin mice respond normally to these agents (Zeller et al. 2005). These results indicate that the respiratory depressant action of etomidate and propofol is largely mediated by β_3-containing $GABA_A$ receptors. Cardiac and respiratory depressant actions of general anesthetics have apparently not been studied in β_2(N265S) mice.

4.1.5 Amnesia

The anterograde amnestic action of propofol was studied in the passive avoidance paradigm and found to be indistinguishable between *β_3(N265M)* knockin mice and wildtype mice, indicating that this effect of propofol is not mediated by β_3-containing $GABA_A$ receptors (Zeller et al. 2007a). Thus, the immobilizing and the anterograde amnestic actions of propofol are mediated by distinct targets. This result is in line with the observation that the anterograde amnestic action of diazepam in the same paradigm is mediated by α_1-containing $GABA_A$ receptors (Rudolph et al. 1999). It is therefore tempting to speculate that the anterograde amnestic action of $GABA_A$ receptor-modulating drugs in

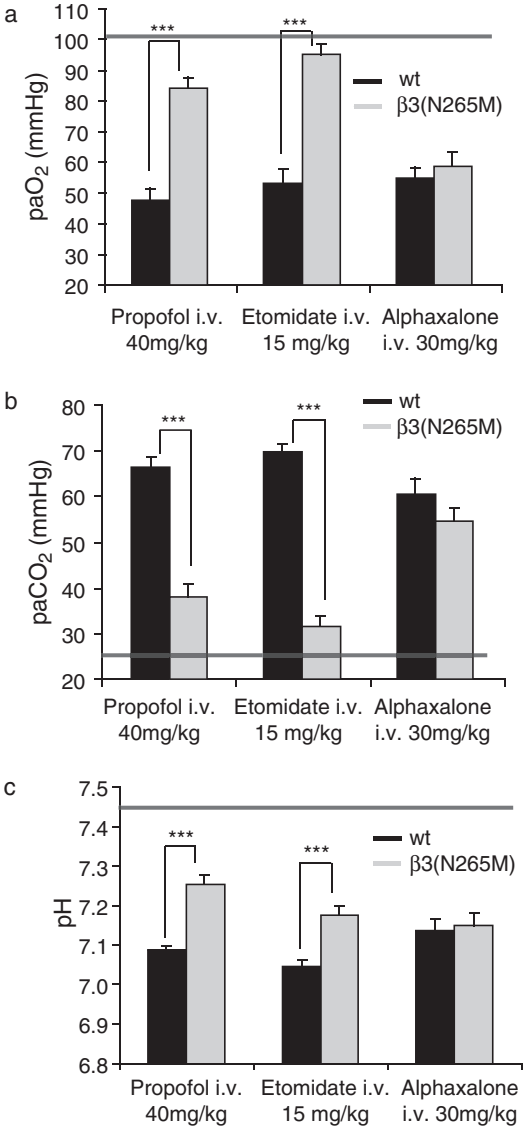

Fig. 2 Assessment of anesthetic-induced respiratory depression by blood gas analysis. **a**, **b** In *β3(N265M)* mice injected with etomidate and propofol, PaO_2 was higher and $PaCO_2$ was lower compared with wildtype mice, indicating the dependence of the respiratory depressant effects of these anesthetics on β_3-containing $GABA_A$ receptors. The neurosteroid anesthetic alphaxalone (mixed in a 3:1 ration with alphadolone, Saffan), whose action is not affected by the $\beta_3(N265M)$ mutation in vitro, elicits changes in blood gases without a difference between genotypes. **c** Similarly, after etomidate and propofol, but not after alphaxalone, pH was higher in $\beta_3(N265M)$ mice compared with wildtype. The *horizontal bars that span the graphs* indicate normal values. n=10; ***$p<0.001$. (Reprinted with permission from *FASEB Journal*, Zeller et al. 2005)

the passive avoidance paradigm is mediated by the most abundant $GABA_A$ receptor subtype, $\alpha_1\beta_2\gamma_2$.

θ-Oscillations (4–12 Hz) are commonly observed during spatial learning and memory tasks. In neocortical slice cultures, local field potentials were recorded and the actions of 0.2 μM etomidate, which causes sedation and amnesia and is approximately 15% of the concentration inducing immobility, were studied. Episodes of ongoing activity occurred spontaneously at a frequency of approximately 0.1 Hz and persisted for several seconds, and toward the end of these periods θ-oscillations developed. In slice cultures from wildtype mice etomidate did not depress θ-oscillations, whereas in slice cultures from *β_3(N265M)* knockin mice θ-oscillations were significantly depressed (Drexler et al. 2005). These results suggest that etomidate has opposing actions on θ-oscillations. These oscillations are enhanced by etomidate acting via β_3-containing $GABA_A$ receptors, and they are decreased by the action of etomidate via receptors other than β_3-containing $GABA_A$ receptors, most likely β_2-containing $GABA_A$ receptors (Drexler et al. 2005). These findings of an opposing action of etomidate on a specific physiological parameter potentially via different $GABA_A$ receptor subtypes have uncovered a so far unrecognized complexity of etomidate action on $GABA_A$ receptors.

The α_5 knockout mice display an improved performance in the Morris water maze compared to wildtype mice in the absence of drugs (Collinson et al. 2002). Moreover, the *α_5(H105R)* knockin mice, which represent a partial α_5 knockout, show increased freezing in trace fear conditioning, which is hippocampus-dependent, but not in delay or context fear conditioning, which is not hippocampus-dependent (Crestani et al. 2002). These results led to the concept that inverse agonists selective for the α_5-containing $GABA_A$ receptor would be suitable as cognitive enhancers (Chambers et al. 2004; Sternfeld et al. 2004). Etomidate was found to decrease freezing in contextual fear conditioning in wildtype mice but not in α_5 knockout mice, and etomidate was also found to impair spatial learning in the Morris water maze in wildtype mice but not in α_5 knockout mice, indicating that α_5-containing $GABA_A$ receptors mediate the actions of etomidate in these tests (Cheng et al. 2006). In these assays, ketamine, a noncompetitive *N*-methyl-d-aspartate (NMDA) receptor antagonist, was equally effective in α_5 knockout and wildtype mice, indicating that the α_5 knockout mice respond normally to agents most likely not acting via the $GABA_A$ receptor system (Cheng et al. 2006). The studies on α_5 knockout mice suggest that the amnestic actions of etomidate are mediated at least in part by α_5-containing $GABA_A$ receptors.

4.1.6 Electrocardiography

Etomidate and propofol increased heart rate variability and prolonged intervals in the ECG (RR, PQ, QRS, QT). All these changes are also seen in β_3(N265M) knockin mice, indicating that these are largely independent of β_3-containing $GABA_A$ receptors (Zeller et al. 2007a).

4.2 Barbiturates

In in vitro studies, barbiturates have a wide range of targets, modulating the activity of $GABA_A$ receptors, nicotinic acetylcholine receptors, *S*-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, and glycine receptors (Krasowski and Harrison 1999), and it is largely unknown which of these ion channels, if any, would mediate the clinical actions of barbiturates. In *β_3(N265M)* knockin mice the barbiturate pentobarbital had no immobilizing action, indicating that the immobilizing action of pentobarbital is mediated by β_3-containing $GABA_A$ receptors (Zeller et al. 2007b). The hypnotic action of pentobarbital is significantly reduced in the *β_3(N265M)* knockin mice, indicating that this action is partially mediated by β_3-containing $GABA_A$ receptors (Zeller et al. 2007b). Thus, with respect to the immobilizing and hypnotic actions, etomidate, propofol, and pentobarbital appear to be dependent on the same drug target, i.e., β_3-containing $GABA_A$ receptors. The respiratory depressant action of pentobarbital was, however, indistinguishable in *β_3(N265M)* knockin mice and wildtype mice, based on the observation that there are no genotypic differences in the *P*aO_2, *P*aCO_2, and pH values (Zeller et al. 2007b). Thus, the respiratory depressant action of pentobarbital is independent of the β_3-containing $GABA_A$ receptors. How can the observation be explained that while pentobarbital clearly binds to β_3-containing $GABA_A$ receptors and the β_3-containing $GABA_A$ receptors can mediate respiratory depression, pentobarbital is respiratory depressant in *β_3(N265M)* mice? The generation of respiratory rhythms occurs in a network of neurons originating from the pre-Bötzinger complex (Richter et al. 2003). Synaptic interactions involving AMPA, NMDA, $GABA_A$, $GABA_B$, and glycine receptors are thought to play a major role in regulating this network. Etomidate- and propofol-induced respiratory depression is mediated by β_3-containing $GABA_A$ receptors, but it is currently unknown which neurons specifically mediate this effect. The observation that pentobarbital induces respiratory depression in *β_3(N265M)* knockin mice indicates that this effect is not mediated by β_3-containing $GABA_A$ receptors or, if it is to some degree, that pentobarbital can also induce respiratory depression via other, currently unknown targets. Both an increase in the inhibitory GABAergic drive and a decrease in excitatory glutamatergic drive can lead to respiratory depression. It is conceivable that pentobarbital might induce respiratory depression by decreasing the glutamatergic drive. This side effect is thus mediated by different receptors or circuits in etomidate- and propofol-induced anesthesia compared to pentobarbital-induced anesthesia. It is tempting to speculate that this mechanistic difference between etomidate and propofol on one hand and pentobarbital on the other underlies the significantly smaller therapeutic range of barbiturates compared to etomidate and propofol.

The hypothermic action of pentobarbital was slightly but significantly diminished in *β_3(N265M)* knockin mice, indicating that this action is mediated to a small extent by β_3-containing $GABA_A$ receptors, but mostly by other targets (Zeller et al. 2007b). Similarly, the heart rate depressant action of pentobarbital is diminished in *β_3(N265M)* knockin mice, suggesting that this action is mediated

both by β_3-containing $GABA_A$ receptors and by other targets (Zeller et al. 2007b). As mentioned previously, we cannot exclude the possibility that the hypothermic action and heart rate depressant action are interdependent.

Pentobarbital increased heart rate variability and ECG intervals (PQ, QT) in both *β_3(N265M)* knockin mice and wildtype mice, suggesting that these actions are largely independent of β_3-containing $GABA_A$ receptors (Zeller et al. 2007b).

4.3 Volatile Anesthetics

The immobilizing action of volatile anesthetics such as isoflurane has been shown to be mediated largely by targets in the spinal cord (Antognini and Schwartz 1993; Rampil et al. 1993; Rampil 1994). The immobilizing response to enflurane, halothane, and isoflurane was moderately decreased in *β_3(N265M)* knockin mice (Jurd et al. 2003; Lambert et al. 2005; Liao et al. 2005) consistent with the hypothesis that the action of these volatile anesthetics are mediated by multiple targets, one of them being β_3-containing $GABA_A$ receptors in the spinal cord. The hypnotic action of these drugs appears to be largely independent of β_3-containing $GABA_A$ receptors (Jurd et al. 2003; Lambert et al. 2005).

A pharmacological study using the $GABA_A$ receptor antagonist picrotoxin suggested that isoflurane-induced immobilization would likely not involve $GABA_A$ receptors (Zhang et al. 2004). This conclusion was largely based on the discrepancy that isoflurane strongly potentiates recombinant $GABA_A$ receptors, in contrast to xenon and cyclopropane, while picrotoxin infusion in the rats increased the EC_{50} for all three anesthetics by approximately 40%. The assumption was that if $GABA_A$ receptors contributed to isoflurane immobilization, picrotoxin should block isoflurane-induced immobilization to a much larger degree than xenon or cyclopropane-induced immobilization. The picrotoxin-induced increase in EC_{50} for xenon and cyclopropane was considered to be unspecific (since the agents apparently do not modulate the $GABA_A$ receptor in vitro), and since the picrotoxin-induced EC_{50} for isoflurane is similar, it was concluded that $GABA_A$ receptors do not mediate isoflurane-induced immobilization. The apparent difference between this study and the study with the knockin mice might be explained by the fact that the pharmacological study may be unable to detect a relatively limited contribution of the $GABA_A$ receptor. Another point to consider is that there is a multitude of $GABA_A$ receptor subtypes, and despite recent advances the exact subunit composition of the $GABA_A$ receptor-mediating immobility is unknown. Thus, the finding that the activity of one or more recombinant $GABA_A$ receptor subtypes is not increased by an anesthetic does not imply that this is true for all $GABA_A$ receptor subtypes expressed in the CNS. Furthermore, recombinant systems lack the natural environment of the $GABA_A$ receptor, and this might have an influence on the responses of this $GABA_A$ receptor to a drug. A recent example of a drug with a discrepancy between its in vitro and in vivo profiles is the anxiolytic ocinaplon, which has no sedative action in

humans, but no selectivity for α_2- or α_3-containing $GABA_A$ receptors (which are presumably mediating anxiolysis) over α_1-containing $GABA_A$ receptors (which are mediating sedation) (Lippa et al. 2005).

In neocortical neurons in cultured slices, enflurane at concentrations between minimal alveolar concentration (MAC)-awake and MAC-immobility depresses spontaneous action potential firing. Enflurane blocks inhibitory postsynaptic current decay and decreases peak amplitudes, thus exerting dual prolonging and blocking effects on $GABA_A$ receptors. In slices from *β_3(N265M)* mice, both prolonging and blocking effects were almost absent, indicating that the β_3(N265M) point mutation essentially abolishes both actions and that β_3-containing $GABA_A$ receptors contribute to the depressant action of enflurane (Drexler et al. 2006).

The hypothermic and heart rate depressant actions of isoflurane have also been found to be slightly but significantly inhibited in *β_3(N265M)* knockin mice compared to wildtype mice, suggesting that these actions are mediated mostly by targets other than β_3-containing $GABA_A$ receptors (Zeller et al. 2007a). Isoflurane increased heart rate variability and prolonged ECG intervals (PQ, QRS, QT) in both wildtype and *β_3(N265M)* knockin mice (with the exception that the increase in the QRS interval was not significant in the mutant mice, possibly due to the small number of animals studied), indicating that these effects are mediated by other targets (Zeller et al. 2007a).

4.4 Ethanol

The targets mediating the effects of ethanol at concentrations as they occur after social drinking have not been identified. Attempts are being made to render individual $GABA_A$ receptor subtypes insensitive to ethanol in recombinant systems and in mice.

In recombinant receptors, the *α_1(S270H)* mutation has been shown to convey insensitivity to isoflurane (Borghese et al. 2006b). This mutation also increases the GABA sensitivity (Borghese et al. 2006b). When it is combined with a second point mutation, α_1(L277A), the GABA sensitivity is near normal in heterologous systems, but the maximal current was decreased (Borghese et al. 2006b), with the current decay time constant higher in wildtype than in α_1(S270H:L277A)$\beta_2\gamma_2$ receptors(Borghese et al. 2006b). Recombinant $GABA_A$ receptors containing the double point mutation are essentially insensitive to modulation by high concentrations of ethanol (Borghese et al. 2006b). In hippocampal CA1 pyramidal neurons, 20 mM and 40 mM ethanol (which might be considered to represent concentrations exceeding those seen with "social" drinking with the legal limit for driving in many jurisdictions being 17.4 mM) increased the $GABA_A$ inhibitory postsynaptic current (IPSC) to the same degree in wildtype mice and mutant mice; however, at 80 mM the increase was substantially reduced in the mutant mice compared to wildtype (Werner et al. 2006). Ethanol-induced hypnosis, locomotor stimulation, cognitive impairment, ethanol preference, and ethanol consumption were indistinguishable in mutant and wildtype

mice (Werner et al. 2006). *α_1(S270H:L277A)* mice are spontaneously hyperactive (Borghese et al. 2006b). They recover more quickly than wildtype mice from the motor impairing action of ethanol and etomidate, but not pentobarbital (Werner et al. 2006). These studies indicate that α_1-containing $GABA_A$ receptors are involved in only a defined subset of ethanol actions.

In recombinant systems, it has been found that the activity of $GABA_A$ receptors containing α_4(or α_6)β_3(or β_2)δ are enhanced by ethanol concentrations as low as 3 mM, whereas the activity of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors are only enhanced by 100 mM ethanol (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003). Other laboratories have been unable to reproduce this finding, suggesting that currently unidentified factors might play a role in ethanol effects at δ-containing $GABA_A$ receptors (Borghese et al. 2006a; Yamashita et al. 2006). Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to $\alpha_4/6\beta_3\delta$ $GABA_A$ receptors (Hanchar et al. 2006), and the low-dose alcohol actions on $\alpha_4\beta_3\delta$ $GABA_A$ receptors are reversed by Ro15-4513 (Wallner et al. 2006), providing further evidence that ethanol might exert some of its effects by interaction with a specific site on a defined $GABA_A$ receptor subtype.

Interestingly, the β_3(N265M) point mutation abolished the effects of high (anesthetic) ethanol concentrations at $\alpha_4\beta_3$(N265M)δ $GABA_A$ receptors, without affecting ethanol enhancement at low doses, suggesting that $\alpha_4\beta_3\delta$ $GABA_A$ receptors have two distinct alcohol modulation sites (Wallner et al. 2006). A R100Q polymorphism in the cerebellar $GABA_A$ receptor α_6 subunit, which increases enhancement of GABA-induced chloride currents in recombinant $\alpha_6\beta_3\delta$ receptors, has been found to enhance granule cell tonic inhibition and to increase alcohol-induced impairment of motor coordination. This suggests that α_6-containing $GABA_A$ receptors in the cerebellum, which are located extrasynaptically, might mediate at least some of the behavioral responses to ethanol (Hanchar et al. 2005).

5 Conclusion

$GABA_A$ receptors have been investigated as molecular targets for the action of a variety of general anesthetics. The intravenous anesthetics etomidate and propofol, as well as pentobarbital, have been shown to exert their immobilizing action and in part their hypnotic action through β_3-containing $GABA_A$ receptors. The proposed roles of β_3-containing $GABA_A$ receptors and other targets for the actions of etomidate and propofol are summarized in Fig. 3. For the immobilizing action of volatile anesthetics, this receptor subtype apparently plays a relatively minor role. While demonstrating a significant role for a specific $GABA_A$ receptors subtype in the action of particular intravenous anesthetics, with respect to volatile anesthetics the data reviewed in this article point to a multisite model of general anesthetic action.

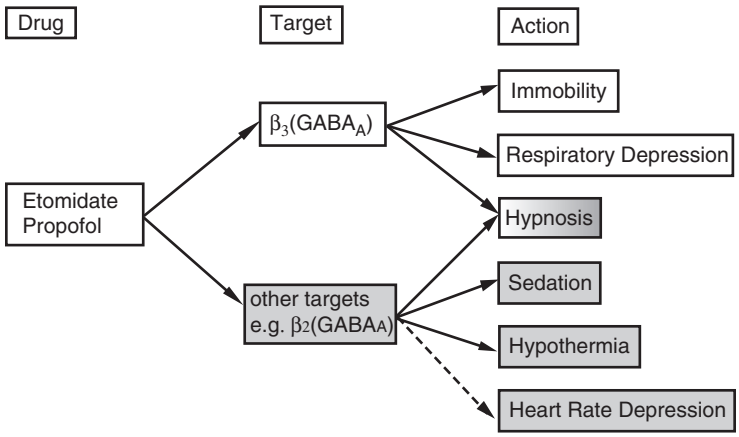

Fig. 3 Proposed roles of etomidate and propofol on $GABA_A$ receptor subtypes. These assignments are based on the following tests: immobility—lost of hind limb withdrawal reflex; respiratory depression—increase in $PaCO_2$ and decrease in PaO_2 and pH; hypnosis—loss of righting reflex; sedation—decrease in motor activity; hypothermia—decrease in core body temperature; cardiac depression—decrease in heart rate. Data are based on this study and previous work. (Reprinted with permission from *FASEB Journal*, Zeller et al. 2005)

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