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THEME ISSUE: MECHANISMS OF ANESTHESIA

Identification and characterization of anesthetic targets by mouse molecular genetics approaches

L'identification et la caractérisation des cibles anesthésiques grâce à des approches fondées sur la génétique moléculaire chez la souris

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Abstract

Purpose *It is now generally accepted that proteins are the primary targets of general anesthetics. However, the demonstration that the activity of a protein is altered by general anesthetics at clinically relevant concentrations in vitro does not provide direct evidence that this target mediates pharmacological actions of general anesthetics. Here we report on advances that have been made in identifying the contribution of individual ligand-gated ion channels to defined anesthetic endpoints using molecular mouse genetics.*

Principal findings *Gamma-aminobutyric acid $(GABA)_A$ receptor subtypes defined by the presence of the α1, α4, α5, β2, and β3 subunits and two-pore domain potassium channels (TASK-1, TASK-3, and TREK) have been discovered to mediate, at least in part, the hypnotic, immobilizing or amnestic actions of intravenous and volatile general anesthetics. Moreover, using tissues from genetically modified mice, specific functions of $GABA_A$ receptor subtypes in cortical and spinal neuronal networks were identified.*

Conclusion *Genetically modified mice have been very useful for research on mechanisms of anesthesia and have contributed to the functional identification of general anesthetic targets and of the role of these targets in neuronal networks.*

Résumé

Objectif *Aujourd'hui, il est universellement accepté que les protéines sont les cibles principales des anesthésiques généraux. Cependant, la démonstration que l'activité d'une protéine est modifiée in vitro par les anesthésiques généraux en concentrations pertinentes d'un point de vue clinique ne constitue pas une donnée probante directe prouvant que cette cible est le médiateur des actions pharmacologiques des anesthésiques généraux. Nous rapportons ici les progrès accomplis dans la détermination de la contribution des canaux ioniques individuels sensibles à un ligand à la définition des critères anesthésiques en utilisant la génétique moléculaire de la souris.*

Constatations principales *On a découvert que les sous-types de récepteurs $GABA_A$ tels que définis par la présence des sous-unités α1, α4, α5, β2, et β3 et les canaux potassiques à deux domaines P (TASK-1, TASK-3 et TREK) médiaient au moins partiellement les actions hypnotiques, immobilisantes ou amnésiques des anesthésiques généraux intraveineux ou volatils. De plus, en analysant des tissus de souris génétiquement modifiées, nous avons pu identifier certaines fonctions spécifiques des sous-types de récepteurs $GABA_A$ dans les réseaux neuronaux corticaux et rachidiens.*

Conclusion *Les souris génétiquement modifiées ont été très utiles à la recherche sur les mécanismes de*

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l'anesthésie et ont contribué à l'identification fonctionnelle des cibles des anesthésiques généraux et du rôle de ces cibles dans les réseaux neuronaux.

Based on the work by Meyer, Overton, and others, it has long been assumed that general anesthetics would exert their pharmacological effects by non-specifically perturbing the neuronal plasma membrane. However, it was later demonstrated in electrophysiological experiments that general anesthetics modulate the activity of a variety of targets, including ligand-gated ion channels, such as gamma-aminobutyric acid $(GABA)_A$ receptors, *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and nicotinic acetylcholine receptors (for review see[1]). These studies have been performed in native tissues or in recombinant systems, the latter allowing the characterization of the sensitivity of defined receptor subtypes to general anesthetics. A potential limitation of the recombinant approach is that there is no guarantee that recombinant receptors behave in the same way as native receptors in a particular assay. *In situ*, neurotransmitter receptor subunits interact with other proteins (e.g., kinases, structural proteins) that may affect functional properties of the corresponding receptor. More importantly, the observation that a given anesthetic agent modulates the activity or directly activates or inhibits a receptor or ion channel target does not provide evidence that the target is essential for the anesthetic actions of the agent. If the target is essential, for which part of the pharmacological spectrum of the general anesthetic is the target responsible?

Genetically modified worms (*C. elegans*) and mice (the focus of this review) have been used to address the question of the functional significance of a specific target for the general anesthetic action of a given drug. A great variety of mouse molecular genetics techniques have evolved over the past two decades. Thus far, the two most important techniques with respect to research on anesthetic mechanisms are (1) targeted gene inactivation ("knockout"), and (2) targeted introduction of a point mutation ("knock-in").

In the targeted gene inactivation ("knockout") approach, a mutation (e.g., deletion of the entire gene, elimination of an essential exon, or insertion of a premature stop codon) is introduced into the gene of interest, which results in the abolishment of the expression of this gene. The basic idea is that if the gene product is essential for any action of a general anesthetic, this action would be missing in the mutant mice. Problems associated with the knockout approach include compensatory regulations that may involve upregulation of related receptor subunits or channels, which may be obscuring the effects of the gene knockout. A case in point are the $GABA_A$ receptor α1 subunit knockout mice. Although it is known from other approaches that α1 plays an important role in mediating the sedative action of benzodiazepines,[2,3] the α1 knockout mice are even more sedated by diazepam than wild type mice,[4] an outcome that may be explained by the upregulation of α2 and α3 subunits in regions where α1 is usually expressed, e.g. the cerebral cortex.[5,6] Another interesting example for a compensatory upregulation is that α6 knockout mice exhibit upregulation of a two-pore domain potassium channel, TASK-1[7] in the cerebellum. In summary, a global knockout mouse may be thought of as having two modifications, i.e., the gene inactivation and the reaction of the animal to the gene inactivation. As a rough guide, if there is a phenotypic difference between knockout and wild type animals, this usually points to the inactivated gene being responsible for the difference; however, no phenotypic difference could mean that the inactivated gene is not involved in the response in question or that it is involved but the compensatory regulations are preserving the wild type phenotype. Sometimes knockout phenotypes may be severe, affecting multiple neuronal systems or the ability to respond in a particular test, and this may also make experiments difficult to interpret.

In contrast, knock-in mice carry more subtle mutations, e.g., single point mutations that block only specific functions of the protein in question. For example, histidine to arginine point mutations in the benzodiazepine binding site of $GABA_A$ receptor α subunits α1, α2, α3, and α5 resulted in the respective $GABA_A$ receptors becoming insensitive to modulation by diazepam, while the response to the physiological neurotransmitter GABA was preserved.[8] Thus, the protein retains substantial functionality, and the expectation is that compensatory secondary changes would not occur or at least they would be substantially smaller than with knockout mice.

Both knockout and knock-in mutations have been generated in order to characterize the functional role of specific neurotransmitter receptors and other ion channels in the action of general anesthetics. Anesthetic endpoints used frequently in rodents are loss of hindlimb withdrawal reflex (or duration of loss of hindlimb withdrawal reflex) or the tail clamp withdrawal reflex (TCWR), which are indicative of immobility and surgical tolerance, or the loss of the righting reflex (LORR), which is used as proxy for unconsciousness (for more detailed description see references 9 and 10). Amnestic endpoints have been addressed using fear conditioning,[11,12] the Morris water maze[11] (a spatial memory task), and the passive avoidance paradigm.[13]

Targets of general anesthetics

$GABA_A$ receptor

The $GABA_A$ receptors are responsible for the majority of fast inhibitory neurotransmission in the central nervous system. The benzodiazepines, which are allosteric modulators of $GABA_A$ receptors containing the α1, α2, α3, or α5 subunits, are widely used as hypnotics, anxiolytics, muscle relaxants, and anticonvulsants. The $GABA_A$ receptors have long been shown to be modulated or even directly activated (although at concentrations that may not necessarily be clinically relevant) by general anesthetics, such as the intravenous anesthetics etomidate, propofol, and pentobarbital, and the volatile anesthetics like isoflurane, enflurane, and halothane (Fig. 1). The $GABA_A$ receptors have also been identified in non-neuronal tissues, e.g., in airway smooth muscle epithelium, where they increase β-adrenergic relaxation of human airway smooth muscle[14] and are involved in mucus overproduction in asthma.[15] This raises the possibility that such non-neuronal $GABA_A$ receptors are contributing to clinical effects of GABAergic drugs or to the phenotypes of global knockout mice.

$GABA_A$ receptor knockout mice

Early work with $GABA_A$ receptor knockout mouse models focused on the immobilizing and hypnotic endpoints. In mice lacking α6-containing $GABA_A$ receptors, it was reported that LORR was unaltered, in response to ethanol, enflurane, and halothane, as was the tail clamp response to enflurane.[16] Lack of δ-containing $GABA_A$ receptors increased the duration of LORR for the neurosteroids, alphaxalone and pregnanolone, but not for etomidate, propofol, midazolam, and ketamine; there was no change in duration of LORR or TCWR with halothane and enflurane.[17] In mice lacking β3-containing $GABA_A$ receptors, the duration of LORR was increased in response to midazolam and etomidate, but not in response to pentobarbital, ethanol, enflurane, and halothane; moreover, the immobilizing action of enflurane and halothane in the TCWR assay was decreased, although the effect size was limited.[18]

Recently, knockout models have been employed to elucidate the amnestic actions of general anesthetics. Studies report 0.10-0.20% incidence of intraoperative awareness among patients, with certain risk groups showing even higher incidence rates.[19,20] Given the large number of anesthesias performed, this frequency translates into approximately 60,000 patients per year in North America.[21] Thus, it is essential to understand the mechanisms underlying the amnestic effects of anesthetics.

Studies report that mice lacking the α5 subunit of the $GABA_A$ receptor exhibit better performance in hippocampal learning tasks compared with wild type mice.[22,23] Electrophysiological studies using slices and organotypic cultures from the hippocampus have reported that GABAergic tonic inhibition and the slow component of the GABAergic inhibitory postsynaptic currents, GABA(A,slow) are enhanced by amnestic concentrations of different anesthetics, e.g., isoflurane,[24] etomidate,[11,25] and propofol.[26] Evidence suggests that these components of GABAergic inhibition are mediated to a great extent by the α5-containing

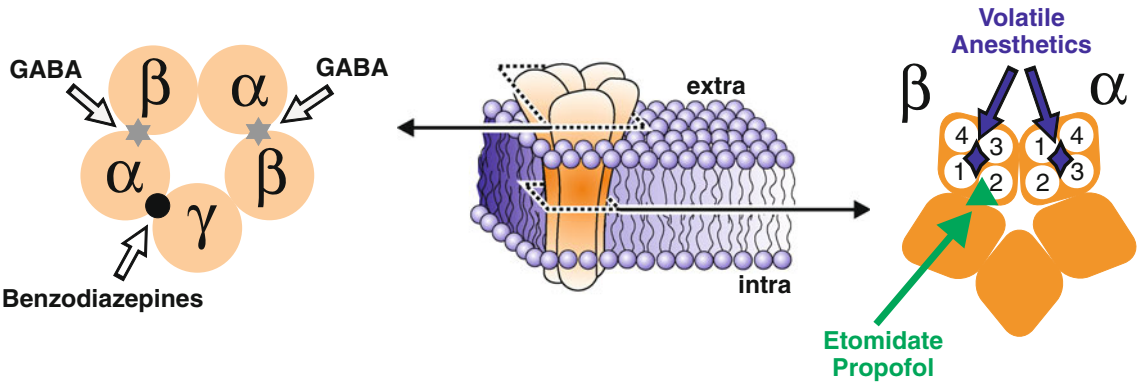


Fig. 1 Pentameric gamma-aminobutyric acid $(GABA)_A$ receptors and binding sites for general anesthetics. The $GABA_A$ receptor is composed of five subunits surrounding the central channel pore. *Central panel*: Positioning of the $GABA_A$ receptor in the lipid bilayer. *Left panel*: Horizontal cross section of the $GABA_A$ receptor as viewed from above, i.e., from the extracellular side. There are two binding sites for the physiological ligand GABA that are located between an α and a β subunit, and there is one binding site for benzodiazepines at the interface of the γ and an α subunit. *Right panel*: Cross section at the level of the plasma membrane. The numbers 1-4 in each of the five receptor subunits depict the transmembrane regions of α subunit. There is strong evidence that the transmembrane region 2 of the β subunit is a component of the binding site for intravenous anesthetics such as etomidate and propofol. As numerous classes of general anesthetics interact with etomidate binding at the $GABA_A$ receptor,[96] this binding site appears to be in close proximity to or overlapping with a binding site of volatile anesthetics. Figure 1 reproduced with permission from[97]

$GABA_A$ receptors in the hippocampus.[27,28] Thus, it is not surprising that the etomidate enhancement of tonic inhibitory currents in CA1 pyramidal neurons was blocked by the application of L-655,708, a α5 $GABA_A$ preferring inverse agonist.[23] Moreover, long-term potentiation (LTP) of field excitatory postsynaptic potentials (EPSPs), a measure widely accepted as a molecular substrate for memory, was reduced by etomidate in hippocampal slices from wild type mice but not from α5 knockout mice.[11]

Behavioural findings from Pavlovian fear conditioning and hippocampus-dependent memory tasks, such as the Morris water maze, corroborate the data from electrophysiological studies. In the Morris water maze, mice treated with etomidate before the training session showed similar acquisition as vehicle-treated mice. However, recall of the platform location 24 hr later was severely impaired in the etomidate-treated animals. Moreover, this reduction in performance following etomidate treatment was not observed in α5 knockout mice.[11,23] Similar findings were reported from Pavlovian fear conditioning studies, where etomidate significantly reduced contextual freezing 24 hr after the acquisition session in wild type, but not in α5 knockout mice. The involvement of α5-containing $GABA_A$ receptors in the effects of etomidate seems to be limited to the amnestic effects. The α5 knockout mice did not show altered sensitivity to the sedative and hypnotic effects of etomidate, as quantified by rotarod, the LORR, and spontaneous motor activity levels.[11]

Similarly, genetic inactivation of the α4 subunit affected LORR minimally and had no effect on immobility in response to isoflurane administration. However, in a conditioned fear experiment, the amnestic action of isoflurane was reduced in α4 knockout animals, implicating α4-containing $GABA_A$ receptors in the amnestic actions of this volatile anesthetic drug.[29]

$GABA_A$ receptor knock-in mice

Specific amino acid residues in the transmembrane domains 2 and 3 of α and β subunits are critical for allosteric modulation of $GABA_A$ receptors by volatile anesthetics.[30] Point mutations have been identified in recombinant systems, which significantly reduce or abolish the action of selected volatile anesthetics while leaving the physiological functions of the receptors largely intact.[30] For example, a double point mutation in the α1 subunit, α1(S270H/L277A) renders α1-containing $GABA_A$ receptors insensitive to isoflurane, but not to halothane.[31] Behaviourally, mice carrying this mutation are more resistant to LORR in response to isoflurane and enflurane, but not to halothane. On the other hand, the immobilizing action of isoflurane, desflurane, and halothane in the TCWR test and the amnestic action of isoflurane in a fear conditioning paradigm were not different between the mutant and the wild type mice, indicating that α1-containing $GABA_A$ receptors play a limited role in the hypnotic action (LORR), but apparently play no role in the immobilizing action (TCWR) and in the amnestic action (fear conditioning).[32] Moreover, etomidate, but not pentobarbital, decreased a smaller amount of time on the rotating rod in the mutant mice than in the wild type mice, indicating an involvement of α1-containing $GABA_A$ receptor in ataxic effects of etomidate.[33]

Naturally occurring $GABA_A$ receptors differ in their sensitivity to etomidate. Studies on the *Rdl*-receptor from Drosophila melanogaster, a close relative of the mammalian $GABA_A$ receptor, revealed that this receptor is insensitive to etomidate.[34,35] In the second transmembrane region, this etomidate-insensitive *Rdl*-receptor carries the amino acid, methionine, at a position corresponding to the asparagine (N265) of the β2 and β3 subunit in etomidate-sensitive $GABA_A$ receptors. Similarly, β1-containing $GABA_A$ receptors, which are an order of magnitude less sensitive to etomidate,[36,37] carry a serine residue at the corresponding position. It was shown that mutation of this asparagine (N) in β2 or β3 to serine (S) or methionine (M) renders $GABA_A$ receptors etomidate-insensitive. While the β2(N265S) mutation rendered receptors insensitive to etomidate but sensitive to propofol, the β3(N265M) mutation rendered receptors insensitive to both etomidate and propofol.[38-40] Mice carrying these two mutations have revealed interesting insights into the contribution of β2- and β3-containing $GABA_A$ receptors to anesthetic responses.

In β2(N265S) mice, the sedative action of subanesthetic doses of etomidate, i.e., the inhibition of locomotor activity, was absent, while the LORR and the pedal withdrawal reflex were still present, although reduced.[41] Moreover, the hypothermic response to etomidate was inhibited in these mice.[42] In contrast, in β3(N265M), the LORR duration in response to etomidate and propofol was reduced, while the hindlimb withdrawal reflex was not lost, indicating that β3-containing $GABA_A$ receptors are indispensible for the immobilizing actions of etomidate and propofol (Fig. 2). The hypnotic actions of these agents, on the other hand, are mediated by both β2- and β3-containing $GABA_A$ receptors.[43] This phenotype is robust, since it was observed on three different genetic backgrounds (129X1/SvJ, C57BL/6J, mixed background).[13] The sedative, i.e., locomotor activity reducing, effect of a subanesthetic dose of etomidate was indistinguishable in wild type and β3(N265M) mice.[44] The hypothermic action of etomidate was moderately reduced in β3(N265M) mice,[44] indicating that β3-containing $GABA_A$ receptors also play a role although this action is largely mediated by β2-containing $GABA_A$

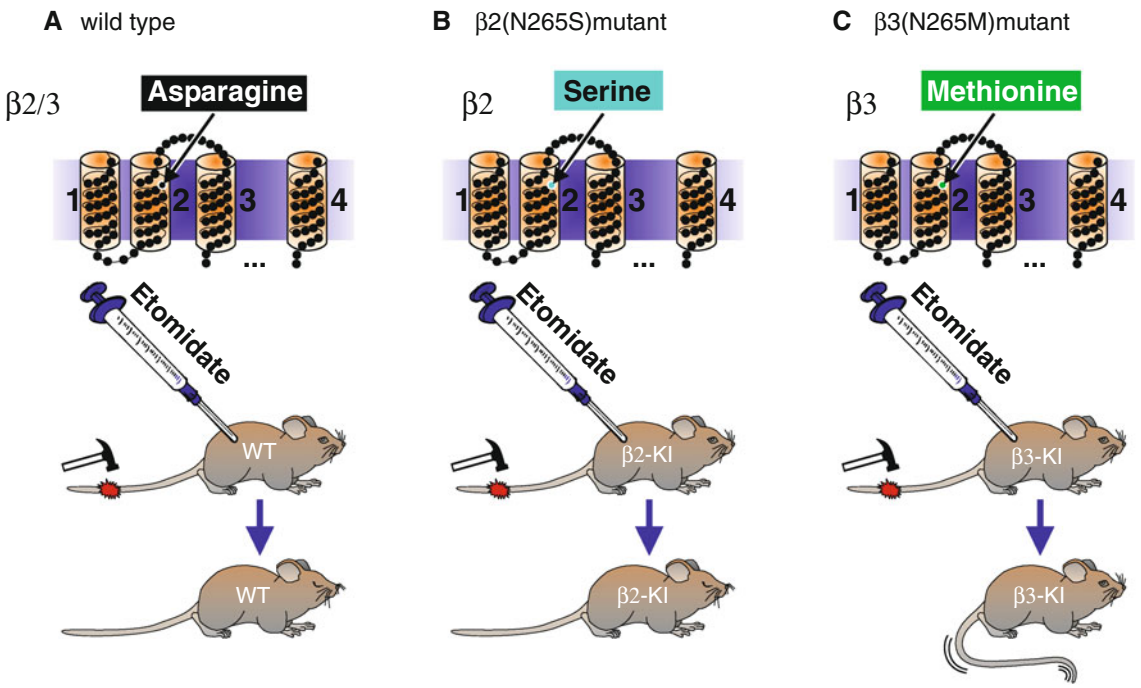


Fig. 2 Immobilizing action of etomidate in wild type, β2(N265S) and β3(N265M) knock-in mice. *Panel A* depicts wild type mice, *panel B* depicts β2(N265S) mice,[41] and *panel C* depicts β3(N265M) mice.[43] At the top of all panels, the β2 or β3 subunit of the gamma-aminobutyric acid $(GABA)_A$ receptor with its four transmembrane domains is shown. A. At position 265, the naturally occurring amino acid in both the β2 and the β3 subunit is asparagine (single letter code N). When a wild type mouse is injected with etomidate, pain reflexes (for illustrative purposes a hammer blow on the tail is shown, while actually paw withdrawal reflexes were assayed) are suppressed, i.e., the animal is immobilized by the general anesthetic. B. By introducing the N265S mutation into the β2 subunit of the $GABA_A$ receptor, this receptor subtype is essentially no longer modulated by etomidate. However, after injection of etomidate, the mouse still does not show a motor reaction in response to a painful stimulus, indicating that β2-containing $GABA_A$ receptors are dispensable for the immobilizing actions of etomidate.[41] C. The N265M point mutation in the β3 subunit of the $GABA_A$ receptor renders β3-containing $GABA_A$ receptors insensitive to modulation by etomidate. After injection of etomidate, the animals still show pain reflexes, demonstrating that $GABA_A$ receptors harbouring β3 subunits are indispensable for the immobilizing properties of etomidate.[43] KI = knock-in. Figure 2 reproduced with permission from[97]

receptors. Etomidate and propofol lead to respiratory depression in wild type mice. In β3(N265M) mice, *P*aCO2 was significantly lower, *P*aO2 was significantly higher, and the pH value was significantly higher than in wild type mice, indicating that the respiratory depression is mediated by β3-containing $GABA_A$ receptors.[44] In contrast, heart rate depression as well as ECG changes were still present in β3(N265M) mice, indicating that they may be mediated by β2-containing $GABA_A$ receptors.[44] The anterograde amnestic action of propofol, as studied in the passive avoidance paradigm, was indistinguishable in β3(N265M) mice and wild type mice, indicating that it is not mediated by β3-containing $GABA_A$ receptors.[13] While the receptor subtype mediating this action is unknown, it is worth noting that the anterograde amnestic action of diazepam has been shown to be mediated, at least in part, by α1-containing $GABA_A$ receptors,[2] presumably of an $\alpha1\beta2\gamma2$ composition.[45] The roles of the β2 and β3 subunits for the major anesthetic actions of etomidate and propofol are summarized in Fig. 3. Results largely similar to those for etomidate and propofol were obtained for the barbiturate pentobarbital, in the β3(N265M) mice, with the major exception that the respiratory depressant action of pentobarbital is present in β3(N265M) mice, indicating that other as yet unidentified receptor targets of pentobarbital can mediate this action.[46]

Finally, the β3(N265M) mutation also inhibits the actions of the volatile anesthetics[40] and has some relatively minor influence on the immobilizing action of halothane, isoflurane, and enflurane, while the mutation has essentially no effect on the hypnotic actions of these agents.[12,43,47]

Glutamate receptors

Glutamate receptors are responsible for a large portion of excitatory neurotransmission in the mammalian nervous system. Ionotropic glutamate receptors (iGluRs) consist of four subunits that form an ion channel. Three categories of

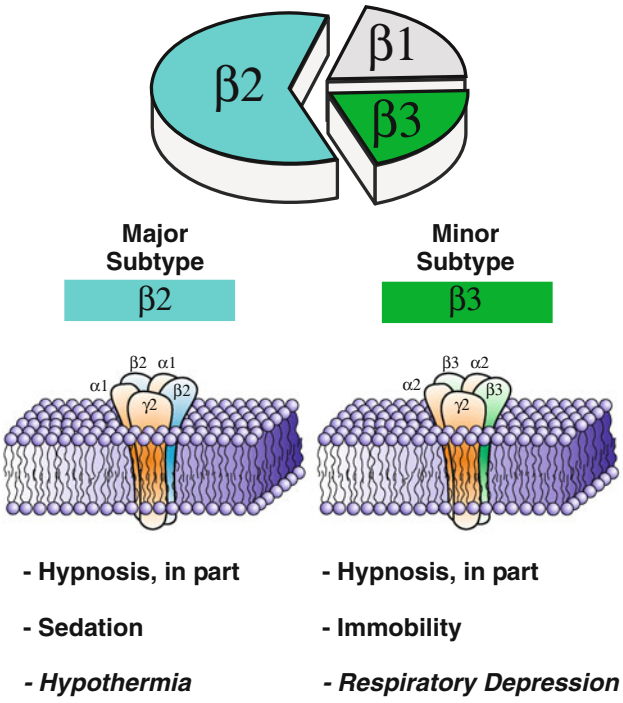


Fig. 3 Contribution of $\beta 2$- and $\beta 3$-containing gamma-aminobutyric acid $(GABA)_A$ receptors to anesthetic endpoints and adverse effects of etomidate and propofol. Figure 3 reproduced with permission from[97]

iGluRs have been defined based on their activation characteristics by different agonists: (1) NMDA receptors, (2) AMPA receptors, and (3) kainate receptors. Metabotropic glutamate receptors (mGluRs) carry transmembrane segments similar to other G-protein coupled receptors and an extracellular ligand binding domain similar to that of iGluRs.[48]

Glutamate-mediated neurotransmission is attenuated by several volatile and gaseous anesthetics.[49,50] This raises the possibility that anesthetic endpoints of at least some compounds are mediated by glutamate receptors. A small body of work has focused on the effects of anesthetics on NMDA and AMPA receptors.

NMDA receptors are tetrameric transmembrane channels composed of an GluN1 subunit (previously also called NR1 or GluRξ1) combined with GluN2 (previously also called NR2 or GluRε) and/or GluR3 subunits.[51] Eight functional splice variants have been noted for the GluN1 subunit,[52] while four types of GluN2 (GluN2A-D) and two types of NR3 (NR3A-B) subunits are encoded by separate genes.[53] Some anesthetics, such as nitrous oxide and ketamine, have minimal effects on $GABA_A$ receptors, but they potently inhibit NMDA receptors.[50,54] Thus, it is possible that the behavioural effects of these anesthetic drugs are mediated, at least partially, by their NMDA-inhibiting actions. A number of studies by Sato *et al.*[55,56] demonstrated that the hypnotic effects of ketamine and nitrous oxide were attenuated in NMDA receptor NR2A subunit knockout mice, specifying the NR2A subunit as one of the targets of these drugs. However, the deletion of the NR2A subunit also dampened the LORR induced by mainly GABAergic agents, such as pentobarbital, diazepam, and midazolam, suggesting that the behavioural phenotype might be due, at least in part, to compensatory mechanisms secondary to the NR2A knockout, which also affect other neurotransmitter systems.

AMPA receptors are tetramers made up of combinations of four subunits: GluA1, GluA2, GluA3, and GluA4 (previously also called GluRA–D, GluR1-4).[57] AMPA receptor properties are predominantly determined by the GluA2 subunit, which conveys calcium permeability of the receptors. GluA2 subunit-containing AMPA receptors are inhibited by barbiturates, whereas the deletion of the GluA2 subunit renders AMPA receptors predominantly insensitive to barbiturate effects.[58] Interestingly, GluA2 knockout mice show increased sensitivity to barbiturate-induced LORR. Moreover, the same kind of hypersensitivity was observed to the effects of volatile anesthetics isoflurane, halothane, sevoflurane, and desflurane, which do not affect AMPA-receptor activity at clinically relevant concentrations, suggesting compensatory mechanisms in GluA2 knockout mice.[59] These findings suggest that caution is required when attributing the changes in behavioural endpoints to receptor-drug interactions. In this case, the increased LORR-sensitivity is likely a result of downstream alterations in other systems caused by the genetic modification rather than the genetically-modified receptor system itself.

Other ion channels with relevance for anesthesia

Two-pore domain background potassium channels modulate neuronal excitability. There are five subunits (TREK-1, TREK-2, TASK-1, TASK-3, and TRESK) known that can homo- and heterodimerize, and channels are found both pre- and postsynaptically. All five subunits can be modulated by halothane, but they are insensitive to clinically relevant concentrations of intravenous anesthetics. In TREK-1 knockout mice, the concentrations of the volatile anesthetics, chloroform, halothane, sevoflurane, and desflurane, required for LORR were increased significantly, although the effect size was small as were the concentrations required for immobilization in the TCWR assay, indicating that TREK-1 plays a limited role in mediating the hypnotic and immobilizing actions of these volatile anesthetics.[60] The concentration of halothane required for LORR was similar in TASK-1 knockout mice and wild

type mice; however, in TASK-1 knockout mice, a higher concentration of isoflurane was required for LORR. Conversely, the concentration of halothane, but not of isoflurane, required for suppression of tail clamp withdrawal was increased.[61] Similarly, TASK-3 knockout mice require higher concentrations of halothane, but not of isoflurane, for suppression of the TCWR.[62] These results indicate a relatively limited but nevertheless significant role of TASK-1 and TASK-3 channels in the hypnotic and immobilizing actions of volatile anesthetics, in line with the idea that volatile anesthetics likely have multiple molecular targets.

Another relevant example of a mouse model providing insights into actions of general anesthetics is provided by TRPA1 knockout mice. In these mice, the volatile anesthetic, desflurane, and the intravenous anesthetic, propofol, fail to excite dorsal root ganglion neurons and thus to activate nociceptive neurons, indicating that TRPA1 channels mediate pronociceptive actions of general anesthetics.[63]

From behavioural endpoints of anesthesia to isolated neuronal networks

The two *in vivo* studies from Reynolds *et al.*[41] and Jurd *et al.*[43] indicated that different endpoints of general anesthetics are mediated by distinct populations of $GABA_A$ receptor subtypes. As these distinct receptor populations are differentially distributed in the central nervous system, this finding also nicely corroborates the idea that individual endpoints of general anesthetics are mediated by neuronal populations in different brain regions. The cerebral cortex has been shown to mediate sedation induced by volatile anesthetics[64] and benzodiazepines,[6] which is in line with the observation that β2-containing $GABA_A$ receptors (contained in the most abundant receptor subtype $\alpha1\beta2\gamma2$) are highly expressed in this brain region. Cortical and subcortical structures mediate hypnosis, and express both β2 and β3.[65] The immobilizing action of general anesthetics, including etomidate and propofol, has been shown to be largely dependent on the spinal cord,[66-69] which displays high expression of β3.[70] The observation that distinct subpopulations of $GABA_A$ receptor subtypes in different parts of the central nervous system mediate different endpoints of general anesthesia, such as hypnosis and immobility, offers the possibility to perform studies using specific isolated networks, e.g., the cortex or the spinal cord. Such studies in isolated networks can bring deeper insights into the mechanisms of anesthesia and are useful *in vitro* systems for the development and testing of new drugs (Fig. 4).

Testing specific neuronal networks *ex vivo*: cortex

As outlined above, selectivity for general anesthetic actions is observed at the molecular and the behavioural level. Thus, it seems reasonable to assume that this might as well be mirrored on the level of neuronal networks. Organotypic slice cultures from the neocortex are one example of an isolated neuronal network. They were introduced by Gähwiler in 1981[71] and have been proven to show *in-vivo*-like morphology, physiology and, most importantly for neuropharmacological studies, $GABA_A$ receptor subtype distribution.[72–75] Moreover, as the diffusion of anesthetics into the tissue is comparatively fast,[76,77] organotypic slice cultures are an ideal tool for investigating anesthetic actions.

Rhythmic electrical activity, i.e., oscillations, is a physiological feature of both *in vivo* and *in vitro* networks. Oscillations in various frequency bands can be recorded from neocortical slice cultures by means of extracellular electrodes (for review see reference 78). Oscillations in the theta range (4-12 Hz) are associated with delayed memory tasks and sensorimotor integration.[79] The generation of theta oscillations depends on rhythmic firing of specific GABAergic interneurons, synchronizing the network.[80]

Etomidate is a positive modulator of GABAergic inhibition, acting predominantly via β2- and β3-containing $GABA_A$ receptors. It is possible that these two subpopulations of $GABA_A$ receptors (those containing β2- and those containing β3-subunits) belong to different inhibitory networks. By using β3 (N265M) point mutant mice, where etomidate actions are largely restricted to β2-containing $GABA_A$ receptors, these networks and their impact on cortical theta oscillations can be experimentally separated. Indeed, cortical theta oscillations were found to be depressed in organotypic slice cultures from the neocortex of β3 (N265M) mice, while no depression was observed in wild type slices.[81] By this experimental separation of the two networks, it was demonstrated that etomidate induces opposing actions on cortical oscillations via different $GABA_A$ receptor populations.

Isolated neuronal networks are also a valuable tool for the investigation of the mechanism of action of general anesthetics. As mentioned above, the two intravenous anesthetics, etomidate and propofol, share their main molecular targets, namely $GABA_A$ receptors harbouring β2- and β3-subunits. While etomidate favours β3 and β2 over β1, propofol is rather unselective in this regard.[36,37] From daily clinical practice, we know that etomidate and propofol have distinct clinical profiles. For instance, etomidate, but not propofol, produces myoclonic movements.[82] Specifically, when used as an anesthetic for electroconvulsive therapy, etomidate increases seizures, while propofol shortens seizures.[83,84] Finally, if sensory-evoked potentials

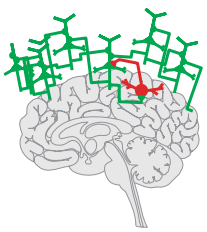
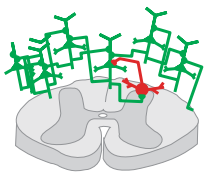
System	Major Findings	Ref.
 **$GABA_A$ receptors**	- Rdl-receptor from Drosophila, although similar to the mammalian $GABA_A$ receptor, is insensitive to etomidate	[34]
	- Etomidate favors β3 and β2 over β1-containing $GABA_A$ receptors, while propofol does not	[36, 37]
	- The interactions of etomidate and propofol with the $GABA_A$ receptor are influenced by one single amino acid	[38, 40]
 **Animals and Behavior**	- β3-containing $GABA_A$ receptors mediate the immobilizing and hypnotic properties of etomidate and propofol	[43]
	- β2-containing $GABA_A$ receptors mediate the sedative properties of etomidate	[41]
	- Respiratory depression by etomidate and propofol are mediated by β3-containing $GABA_A$ receptors	[44]
	- β3-containing $GABA_A$ receptors mediate the immobilizing, part of the hypnotic, but not the respiratory depression by pentobarbital	[46]
	- Etomidate-induced hypothermia is mediated mainly by β2-containing $GABA_A$ receptors	[42, 44]
	- Etomidate induced amnesia is mediated by α5-containing $GABA_A$ receptors	[11]
 **Neuronal Networks** 	- Etomidate induces opposing effects on cortical theta oscillations via different $GABA_A$ receptor subtypes	[81]
	- Etomidate and propofol show distinct actions at β3-containing $GABA_A$ receptors	[87]
	- The tuberomammillary nucleus and the perifornical area, both being parts of natural sleep pathways, are important targets of propofol	[91]
	- β3-containing $GABA_A$ receptors are largely contributing to depressant actions of etomidate in neuronal networks of the ventral horn of the spinal cord	[92]

Fig. 4 Important findings from molecular genetic studies with intravenous anesthetics

are used for monitoring purposes, etomidate enhances signal amplitude, but propofol does not.[85,86] But how can we explain these evidently different clinical profiles of etomidate and propofol considering their largely overlapping molecular targets, in particular $\beta 2$- and $\beta 3$-containing $GABA_A$ receptors? One possible explanation can be found

in their effects on the firing patterns of cortical networks. A typical firing pattern of isolated cortical networks is characterized by bursts of action potential firing separated by neuronal silence. Etomidate and propofol were shown to have differential effects on this typical firing pattern. Etomidate shortened the bursts and reduced the number of action potentials per bursts. These effects were absent in $\beta 3$ (N265M) mutant slices, indicating that these actions are mediated by $\beta 3$-containing $GABA_A$ receptors. In contrast, propofol increased burst length.[87]

The differential effects of etomidate and propofol are also detectable at the synaptic level. The actions of GABAergic synapses can be recorded as inhibitory postsynaptic currents. An inhibitory postsynaptic current is characterized by an initial very steep rising phase, a first phase of rapid current decay and a second phase of slow current decay. In cortical slice cultures, etomidate exclusively affected the slow phase, while propofol had effects on both phases of the current decay.[87] These results indicate the following: (1) the differential clinical effects of etomidate and propofol are mirrored on the level of the cortical network and also on the level of the GABAergic synapse; and (2) as the effects of both drugs were qualitatively different in slices from $\beta 3$ (N265M) mutant mice compared with wild type mice, they can be attributed to different subpopulations of $GABA_A$ receptors. These results support the view that differences in the molecular interactions of anesthetics with their targets lead to distinguishable differences in network firing effects, which finally translate into distinct clinical profiles (Fig. 5).

Besides cortical networks, subcortical structures are also important targets for general anesthetics.[88,89] In particular, endogenous sleep pathways appear to play a prominent role.[90] In support of this hypothesis, studies using $\beta 3$(N265M) knock-in mutant mice showed that the effect of propofol on the decay time of GABAergic currents was dependent on $\beta 3$-containing $GABA_A$ receptors in the tuberomammillary nucleus and the perifornical area, but not in the locus coeruleus. Thus, the $\beta 3$-containing $GABA_A$ receptors in the tuberomammillary nucleus and the perifornical area, but not in the locus coeruleus, may be direct targets for the hypnotic action of propofol.[91]

Testing specific neuronal networks *ex vivo*: spinal cord

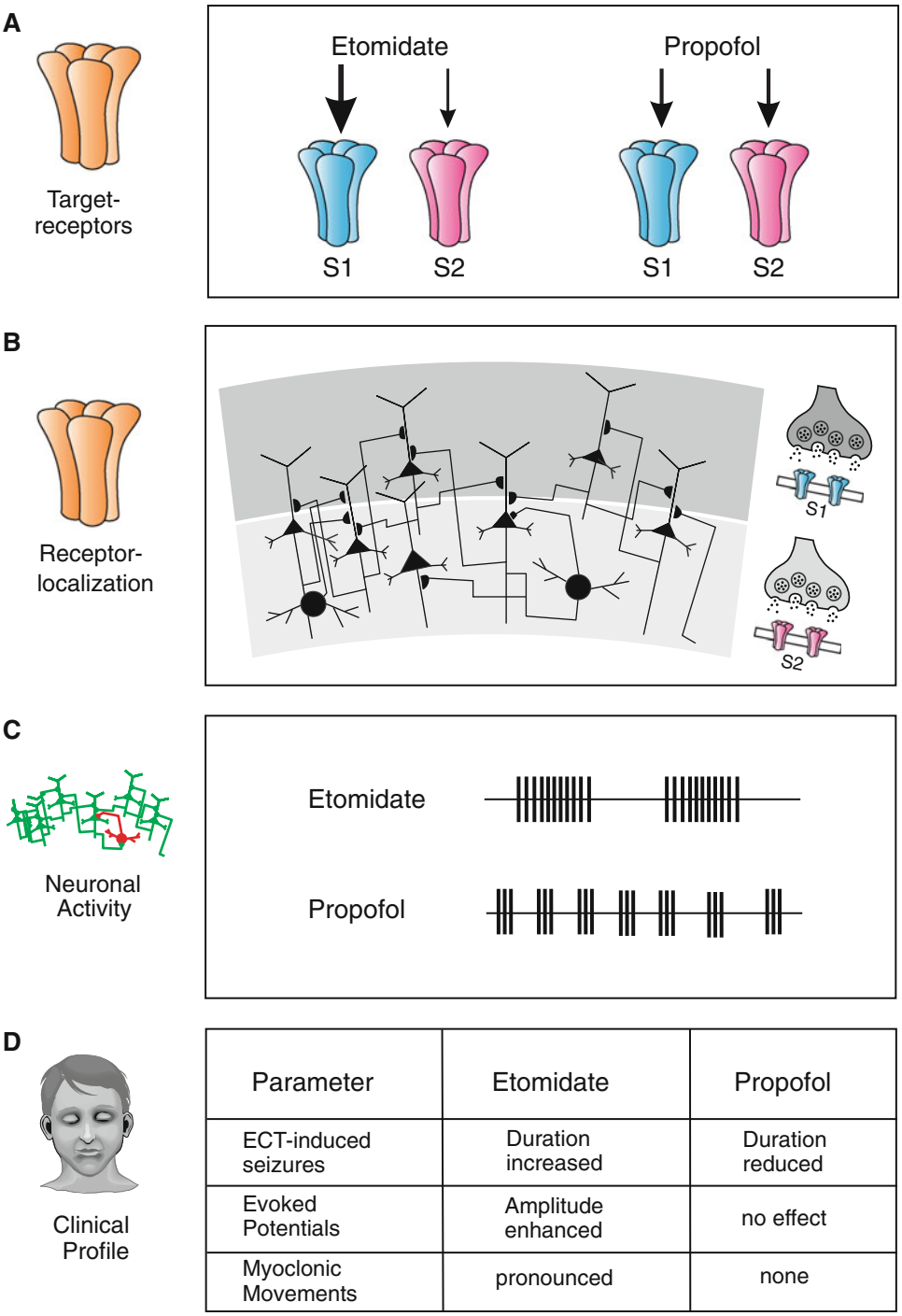
The spinal cord is probably the most important part of the central nervous system mediating the general anesthetic endpoint immobility.[66-69] As mentioned before, in $\beta 3$(N265M) mutant mice, the immobilizing actions of etomidate and propofol were abolished.[43] Thus, it was not surprising that the network-depressing effects of etomidate were also reduced significantly in cultured spinal cord slices from these knock-in mice. However, using cultured spinal cord slices from $\beta 3$ (N265M) mutant mice, the authors demonstrated that (1) the depression of ventral horn neurons by etomidate is limited to approximately 60%; and (2) this limitation is most likely based on the fact that etomidate also depresses presynaptic GABA release.[92] As etomidate action (positive modulation of GABAergic inhibition) most likely depends on the presence of the physiological neurotransmitter GABA, this means that etomidate literally "bites the hand that feeds it" by depressing presynaptic GABA release, resulting in only partial depression of ventral horn neurons. As this presynaptic effect of etomidate was absent in $\beta 3$ (N265M) mutant spinal slices, it can be concluded that this self-restrictive and, hence, undesired effect of etomidate is mediated by $\beta 3$-containing $GABA_A$ receptors that are presynaptically located.[92] This example illustrates that electrophysiological studies of isolated neuronal networks using tissues derived from knock-in mice are an elegant approach for investigating mechanisms of action of general anesthetics and also for testing the immobilizing properties of novel compounds.[93]

Multiple targets of volatile anesthetics

As mentioned previously, the volatile anesthetics interact with multiple molecular targets, including $GABA_A$ receptors.[9] For electrophysiological studies on $GABA_A$ receptors, perhaps the best model substance is enflurane, because it displays most prominently the typical effects of volatile anesthetics on inhibitory postsynaptic GABAergic currents, i.e., prolongation of decay time and reduction of the current amplitude. Distinct mechanisms have been suggested to underlie these two actions.[94] Both actions of enflurane were abolished in cortical slice cultures from $GABA_A$ receptor $\beta 3$ (N265M) mutant mice, demonstrating that $\beta 3$-containing $GABA_A$ receptors are involved in both mechanisms.[95]

Clinical implications

A crucial factor in choosing a drug for inducing anesthesia in a specific clinical setting is the pattern of potential adverse side effects. In fact, all anesthetic agents in current use display a long list of unwanted actions, including cardiovascular instability, hypothermia and postoperative shivering, respiratory depression, postoperative vomiting, and nausea. The molecular and cellular mechanisms contributing to these side effects remain to be elucidated. Interestingly, studies on genetically modified mice provided first evidence that certain $GABA_A$ receptor subtypes



Parameter	Etomidate	Propofol
ECT-induced seizures	Duration increased	Duration reduced
Evoked Potentials	Amplitude enhanced	no effect
Myoclonic Movements	pronounced	none

Fig. 5 Comparison of the properties of etomidate and propofol at different levels of observation. A. The intravenous anesthetics, etomidate and propofol, have largely overlapping gamma-aminobutyric acid $(GABA)_A$ receptor targets (etomidate: $\beta 2$, $\beta 3$; propofol: $\beta 1$-$\beta 3$).[36,37] The differently sized arrows symbolize that propofol apparently has no preference, while etomidate has some preference for $GABA_A$ receptor subpopulations ("S1", "S2"). B. Different subtypes of $GABA_A$ receptors display different regional and cellular localizations, physiological, and pharmacological properties,[98-100] e.g., while one subpopulation might be predominant in the upper layers of the cerebral cortex, it might be only a minor subtype in deeper layers and *vice versa*. This results in a differential representation of receptor subpopulations in neuronal networks. C, D. The combination of the differences described in A and B (i.e., preference of $GABA_A$ receptor subpopulation "S1" by etomidate and equal action of propofol at "S1" and "S2") together with the differential localization of the two receptor subpopulations within a neuronal network leads to differential electrophysiological network effects[87] and clinical effects of etomidate and propofol

mediate unconsciousness and immobility, whereas others are involved in causing cardiovascular depression and hypothermia.[43,44] These findings suggest that the desired and undesired clinical actions of anesthetics are mediated, at least in part, by different molecular targets. Thus, improving the selectivity of anesthetic agents may open new avenues for the development of new compounds with an improved side effect profile. Indeed, there is a need for improved anesthetic compounds, in particular for high-risk populations as well as in very young and very old patients.

Conclusions

Research on genetically modified mice, e.g., knockout and knock-in mutants, has led to significant progress in the identification and functional characterization of the

anesthetic targets in the central nervous system. These investigations define the scientific basis for the rational design of improved anesthetic compounds in the future. Furthermore, combined *in vitro* and *in vivo* studies provide important clues about the specific functions of neural networks and ion channels during wakefulness, sleep, and general anesthesia.

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Conflict of interest None declared.

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