Handbook of Experimental Pharmacology 182

Jürgen Schüttler
Helmut Schwilden
Editors

Modern Anesthetics

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Modern Anesthetics

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Preface

Some important constraints of anesthesia must be taken into consideration when the pharmacological properties of modern anesthetics are discussed. The most important of these could be that the target effect be achieved preferably within seconds, at most within a few minutes. Similarly, offset of drug action should be achieved within minutes rather hours. The target effects, such as unconsciousness, are potentially life-threatening, as are the side effects of modern anesthetics, such as respiratory and cardiovascular depression. Finally, the patient's purposeful responses are not available to guide drug dosage, because, either the patient is unconscious, or more problematically, the patient is aware but unable to communicate pain because of neuromuscular blockade.

These constraints were already recognised 35 years ago, when in 1972 Volume XXX entitled "Modern Inhalation Anesthetics" appeared in this Handbook Series. The present volume is meant as a follow up and extension of that volume. At the beginning of the 1970's anesthesia was commonly delivered by inhalation, with only very few exceptions. The clinical understanding of that time considered anesthesia as a unique state achieved by any of the inhalation anesthetics, independent of their specific molecular structure. "The very mechanism of anesthetic action at the biophase" was discussed within the theoretical framework of the "unitary theory of narcosis". This theoretical understanding was based on the Meyer-Overton correlation and the apparent additivity of MAC when several inhalational anesthetics were given simultaneously, MAC being the measure of anesthetic potency and anesthetic depth developed in the mid-1960's. Since the 1980's this understanding has changed completely. Today "general anesthesia" is commonly considered a collection of neurophysiologically very different states, achieved by a multitude of very different drugs (delivered not only by inhalation) acting on a plethora of subcellular structures. Unconsciousness and absence of pain are always included in this collection of different states.

Three main factors contributed to this changed understanding:

1) the increasing use of intravenous anesthesia, facilitated by the development of new intravenous anesthetics, not only for the induction but also for the maintenance of anesthesia
2) the discovery of non-additive types of anesthetic interactions,

3) the development of molecular techniques (biological, pharmacological and physiological) to study the interaction of anesthetic drug molecules with receptive cell structures.

For these reasons, when the outline of this Handbook was discussed at a brainstorming meeting in Erlangen in February 2005, it became clear that it should be entitled "Modern Anesthetics" and contain in addition to a section on "Inhalation Anesthetics" one on "Intravenous Anesthetics", preceded by another on "Molecular Mechanisms of Anesthetic Action". Emphasis was put on the term "molecular" to draw attention to the discovery in the past decades of a great many findings on the interaction of anesthetic compounds with subcellular entities. On the other hand, this emphasis was to underline the lack of our understanding concerning the summation of all the different interactions from the molecular level through the progressive stages of integration within the CNS, which needs to be studied in the future. While these considerations may be considered mainstream of current research in experimental anesthetic pharmacology, it was strongly felt that the particularities of anesthetic drug therapy discussed above require not only specific drugs, but also very particular modes of their delivery and administration. It is not only the properties of the compounds but the combination of compounds plus drug delivery system which turns the compounds into a clinically effective and safe drug. It was therefore thought necessary to integrate a fourth section on "Pharmacokinetics-Pharmacodynamics based Administration of Anesthetics". This final section illustrates a strategy, still at an experimental stage, in which the integration of drug, medical technology and computational medicine leads to optimized anesthetic therapeutic systems.

We wish to thank all colleagues and authors for their endurance and willingness to contribute all their efforts and a considerable amount of time, to share freely their outstanding expertise and knowledge for this Handbook. Special thanks go to those who took responsibilities for each of the four sections: to Bernd Urban for "Molecular Mechanisms of Anesthetic Action", to Jim Bovill for "Modern Inhalation Anesthetics", to Frederic Camu for "Modern Intravenous Anesthetics", and to Don Stanski for "Phamacokinetics-Pharmacodynamics based Administration of Anesthetics".

Erlangen, Germany

Jürgen Schüttler
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Part I
Molecular Mechanisms of Anesthetic Action

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The Site of Anesthetic Action

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Abstract The mechanisms of general anesthesia constitute one of the great unsolved problems of classical neuropharmacology. Since the discovery of general anesthesia, hundreds of substances have been tested and found to possess anesthetic activity. Anesthetics differ tremendously in their chemical, physical, and pharmacological properties, greatly varying in size, in chemically active groups, and in the combinations of interactions and chemical reactions that they can undergo. The

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large spectrum of targets makes it obvious that dealing with anesthetics pharmacologically is different from dealing with most other drugs used in pharmacology. Anesthetic potency often correlates with the lipophilicity of anesthetic compounds, i.e., their preference for dissolving in lipophilic phases. This suggests as a main characteristic of anesthetic interactions that they are weak and that for many of them there is overall an approximate balance of nonspecific hydrophobic interactions and weak specific polar interactions. These include various electrostatic (ions, permanent and induced dipoles, quadrupoles), hydrogen bonding, and hydrophobic interactions. There are many molecular targets of anesthetic action within the central nervous system, but there are many more still to be discovered. Molecular interaction sites postulated from functional studies include protein binding sites, protein cavities, lipid/protein interfaces, and protein/protein interfaces.

1 Introduction

The mechanisms of general anesthesia remain one of the great unsolved problems of classical neuropharmacology (Miller 1985). Definitions, concepts, and hypotheses concerning general anesthesia have been discussed at length elsewhere (Urban and Bleckwenn 2002; Urban 2002; Campagna et al. 2003; Sonner et al. 2003; Rudolph and Antkowiak 2004; Franks 2006; Evers and Crowder 2005; Koblin 2005). Since there is no agreement on the mechanisms of general anesthesia, sites for interactions of general anesthetics will be discussed without attempting to decide whether or not they are relevant for general anesthesia.

The first section will review which drugs produce general anesthesia both clinically and experimentally, and which targets they affect. The next section will describe the molecular interactions that anesthetics are capable of undergoing with their targets. The final section will discuss molecular sites of anesthetic actions that have been investigated in detail.

2 Anesthetics and Their Targets

Since the discovery of general anesthesia hundreds of substances have been tested and found to possess anesthetic activity (Urban et al. 2006). Only very few of these have ever been introduced into clinical practice. The ability of an anesthetic drug to produce experimental general anesthesia is a necessary but not a sufficient condition for its use in humans. It is their side effects that rule out most general anesthetics for clinical use.

2.1 General Anesthetics in Clinical Use

Only a few anesthetics are listed by Goodman and Gilman's *The Pharmacological Basis of Therapeutics* (Hardman et al. 2001) as being used in clinical practice

today. They comprise the halogenated ethers sevoflurane, desflurane, isoflurane and enflurane, the halogenated alkane halothane, nitrous oxide, a few barbiturates, a few benzodiazepines, etomidate (imidazole derivative), propofol (phenol derivative), ketamine (phencyclidine derivative), and the opioid analgesics (Fig. 1). While the use of halothane, enflurane, and nitrous oxide is clearly declining, the noble gas xenon is about to be introduced into clinical practice. Barbiturates serve mainly as agents for induction of anesthesia. Opioids are predominantly used as analgesics. Although their use as general anesthetics is controversial (Hug 1990), as an adjuvant they help to reduce the amount of other anesthetic agents needed.

Most of these compounds, however, be they modern halogenated inhalation anesthetics or intravenous anesthetics, cannot be used by themselves as universally as diethyl-ether once was. For example, the intravenous anesthetic ketamine is not

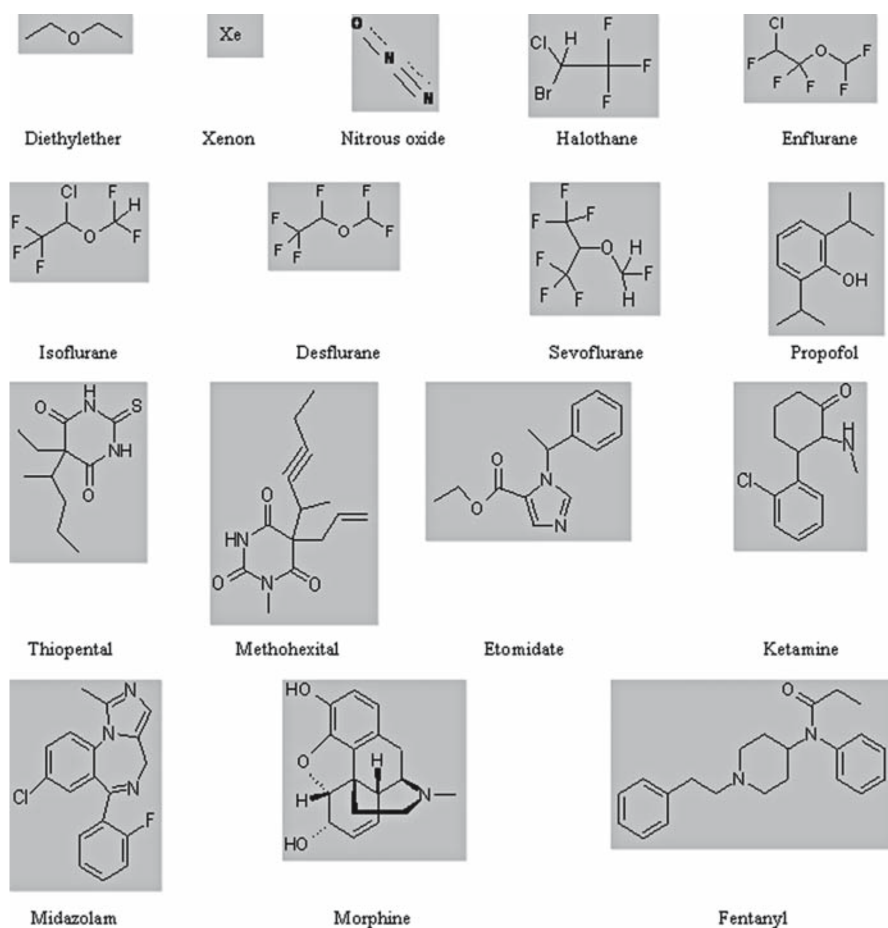

Fig. 1 Anesthetics and anesthesia adjuvants widely used in clinical practice, except for diethyl ether, which is shown for historical reasons

given by itself, but is commonly co-administered with benzodiazepines to counteract the possible undesirable psychological reactions which occur during awakening from ketamine anesthesia (Reves et al. 2000). Almost all halogenated ethers such as isoflurane or desflurane lack sufficient analgesic potency and may even possess hyperalgesic properties (Antognini and Carstens 2002). Intravenous anesthetics such as barbiturates or propofol also lack analgesic potency. Modern general anesthetic techniques in clinical use typically involve the co-administration of a hypnotic drug, an analgesic drug, and possibly a muscle relaxant, allowing the reduction of hypnotic drug concentrations and thereby reducing side effects.

2.2 General Anesthetics in Experimental Use

Hundreds of substances have been examined as general anesthetics in experimental anesthesia (Adriani 1962; Seeman 1972; Lipnick 1991; Miller 2004; Urban et al. 2006). Volatile and nonvolatile anesthetics form two major divisions of anesthetic compounds. On the whole, the volatile drugs are relatively inert molecules that are mostly nonreactive in the body. The nonvolatile drugs, on the other hand, tend to be reactive and are subject to modification by biochemical mechanisms. Anesthetics differ tremendously in their chemical, physical, and pharmacological properties, greatly varying in size, and in chemically active groups. Quite possibly the anesthetics are the most heterogeneous class in all of pharmacology. The large spectrum of targets makes it obvious that dealing with anesthetics pharmacologically is different from dealing with most other drugs used in pharmacology.

2.3 Anesthetic Potency

All clinical measures of anesthetic potency are but surrogate measures. The clinically most prevalent measure of anesthetic potency is MAC (minimal alveolar concentration). It measures the end-tidal concentration of inhaled anesthetic that suppresses purposeful movement in response to surgical incision in 50% of a test population (Eger et al. 1965). It has now become clear that MAC reflects more of a spinal than a cortical response (Antognini and Carstens 2002).

MAC and movement responses to noxious stimuli are no longer as useful in clinical practice because of the extensive use of muscle relaxants. It has become clear that clinical anesthetic potency has to be quantified separately for the different components of general anesthesia such as consciousness, amnesia, analgesia, or reflex activities. Different physiological responses have been tried as alternatives to monitor adequate anesthesia: heart rate, arterial pressure, the rate and volume of ventilation in spontaneously breathing subjects, eye movement, the diameter and reactivity of pupils to light, and other autonomic signs such as sweating (Stanski and Shafer 2004). Using a combination of some of these parameters, Evans (1987)

developed the PRST score (pressure, heart rate, sweating, tear production) that, however, is not widely used. Spontaneous electroencephalograms (EEG) and evoked potentials (EG) are electrical brain activities that have been employed to quantify the hypnotic component (Stanski and Shafer 2004).

Stanski criticized the fact that clinical measures with poor or unpredictable utility when evaluated scientifically (blood pressure or pulse) have become the mainstay of clinical assessments of depth of anesthesia in routine clinical practice (Stanski and Shafer 2004). It still remains true today that no numerical measure of clinical potency and no monitor, but rather many years of experience, will tell an anesthesiologist whether or not a patient is adequately anesthetized. The only “hard numbers” available at present are either MAC values and their equivalent Cp_{50} values for intravenous agents (Glass et al. 2004) or empirical doses and concentrations recommended by textbooks and typically given in the operating rooms.

The importance of carefully defining functional endpoints when assessing anesthetic potency of in vivo or in vitro experiments has been discussed elsewhere (Urban et al. 2006); there is also a need to establish complete concentration-response curves for each functional endpoint.

2.4 *Identifying Molecular Targets*

As the publications from the most recent Seventh International Conference on Molecular and Basic Mechanisms of Anaesthesia and previous conferences (Fink 1975; Fink 1980; Roth and Miller 1986; Rubin et al. 1991; Richards and Winlow 1998; Urban and Barann 2002; Mashimo et al. 2005) have shown, there are a great many molecular targets of anesthetic actions within the central nervous system. While in the past much attention has focused on ion channels, other proteins have been found to be sensitive to anesthetics as well (Urban et al. 2006). Currently under investigation and definitely of interest are, for example, metabotropic receptors, which modulate synaptic transmission and partly bind the same ligands as ligand-gated ion channel receptors. Other proteins affected by anesthetics are protein pumps, G proteins, protein kinases, and phosphatases, as well as adrenergic receptors, prostanoid receptors, motility proteins, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, or fatty acid amide hydrolase (FAAH) (Urban et al. 2006).

Still, relatively speaking, the anesthetic sensitivities of only a few proteins have been investigated, when compared with the estimated number of at least 12,000 different membrane proteins, of which ion channels are only a small fraction. The known list of molecular anesthetic targets (Urban et al. 2006) is steadily increasing as ongoing research on other molecular targets is constantly revealing new targets.

There is a great deal of discussion and dissent on which molecular targets and which molecular mechanisms are relevant for general anesthesia (Urban and Bleckwenn 2002; Urban 2002; Campagna et al. 2003; Sonner et al. 2003; Rudolph

and Antkowiak 2004; Franks 2006; Evers and Crowder 2005; Koblin 2005). This is perhaps not surprising since many levels of integration within the central nervous system have to be passed before an anesthetic action at the molecular level is sensed at the systemic level. As long as the detailed architecture of these pathways and networks remains mostly obscure, a final judgment on the relevance of molecular anesthetic targets should be postponed.

Several points can be made by surveying the existing information on anesthetic actions on the molecular targets: (1) Even at clinical concentrations, anesthetics act on many different molecular targets. (2) Wherever investigated in detail, it has been found that any single anesthetic suppresses proteins by more than one action, i.e., anesthetics affect not just one but several different aspects of any particular molecular target. (3) No two anesthetics appear to act alike on the same target; they all have their individual spectra of effects. (4) Anesthetics differ not only quantitatively in the relative strengths of their various effects but also qualitatively, in that both suppression as well as potentiation may occur.

3 Physical and Chemical Nature of Anesthetic Interactions

Two fundamentally different approaches have been used in order to characterize interactions between anesthetics and their targets: thermodynamic descriptions and molecular descriptions. Thermodynamic descriptions consider averages over many individual interactions, while molecular descriptions attempt to measure directly individual interactions between anesthetic molecules and their molecular targets. The thermodynamic approach has been largely replaced by molecular approaches as increasingly refined molecular methods have become available to investigate interactions between anesthetics and their targets.

3.1 Thermodynamic Approaches

3.1.1 Solution Theories

Although anesthetically active substances may vary greatly in size and in other physical and physiochemical properties—not to mention their pharmacological behavior—they do have something in common. More than 100 years ago Meyer and Overton independently discovered that anesthetic potency correlated with the preference of anesthetics to dissolve in lipophilic rather than in polar phases (Urban et al. 2006). They found a linear relationship between the logarithm of anesthetic potency and the logarithm of the oil/water partition coefficients, with unity slope, now called the Meyer-Overton correlation (Urban et al. 2006).

The Meyer-Overton correlation was found long before the concept of cell membranes existed, and the researchers therefore concluded that anesthesia

was brought about by anesthetics dissolving the lipophilic moieties of a cell. A thermodynamic description of solutions and interactions of solutes with solvents was used in order to describe anesthetic action. These descriptions of anesthetic interactions could be easily transferred to membranes once their concept had been established. Anesthetic potencies were described in terms of chemical potentials partitioning between different solvents and various solubility parameters (Butler 1950; Ferguson 1939; Kaufman 1977; Mullins 1954; Hildebrand and Scott 1964). These descriptions did not concentrate so much on how these interactions brought about anesthesia. Instead, they sought to identify parameters that would predict at what concentration any given substance would produce anesthesia.

3.1.2 Meyer-Overton Rule

To date, no other rule based on physiochemical or structural parameters has been as useful as the Meyer-Overton rule in predicting anesthetic potency. The knowledge of the partition coefficient of a substance is in most cases sufficient to predict its anesthetic potency quite accurately, provided the substance is chemically not too complex (Urban et al. 2006).

When anesthetic potency data collected from various in vivo and in vitro systems were plotted against the same consistent set of octanol/water partition coefficients, comparison of the resulting different lipophilicity plots led to the following observations (Urban et al. 2006). First, different classes of anesthetics give rise to different correlations that are shifted with respect to each other. Second, intravenous anesthetics are, on the whole, considerably more potent than inhalation anesthetics. Third, different proteins may differ in their sensitivities to anesthetics, depending on the group of anesthetics involved.

The macroscopic Meyer-Overton rule does not provide any direct microscopic insight. However, the existence of so many Meyer-Overton correlations appears to imply that the hydrophobic component of the anesthetic interaction is roughly equal to weak polar components and therefore is not being masked by them (Urban et al. 2006). Consistent with anesthetic interactions being weak is the observation that IC_{50} values in the millimolar and micromolar range are characteristic in general anesthesia, and that large quantities of anesthetic drugs (in the order of grams or at least milligrams) have to be administered during inhalation anesthesia and intravenous anesthesia (barbiturate, propofol, ketamine, etomidate).

3.1.3 Multiple Linear Regression Analyses of Various Physical Properties

Without examining hydrophobic and weak polar interactions directly on the molecular level, attempts have been made to identify their contributions by using multiple linear regression analysis on thermodynamic parameters. Equations similar to the following have been used to quantify the relative contributions of various

physical properties of an anesthetic (i.e., its ability to donate or accept a hydrogen bond, its dipolarity and polarizability, and its size) to the magnitude of partition coefficients or concentrations of anesthetic endpoints (Abraham et al. 1991; Davies et al. 1974):

$$\log (P) = c + s\cdot\pi + d\cdot\delta + a\cdot\alpha + b\cdot\beta + \nu\cdot V$$

where *P* is the partition coefficient between two solvents or the potency of an anesthetic. The solute parameters in this equation represent the following interactions: π, the solute dipolarity/polarizability; δ, a polarizability correction term; α, the solute (hydrogen-bond donor) acidity; β, the solute (hydrogen-bond acceptor) basicity; and *V*, the solute volume. Solute volume is so closely correlated with lipophilicity (or hydrophobicity) that the coefficient (ν) of *V* can be considered to be a measure of the lipophilicity of the condensed phase being investigated. The constants *c*, *s*, *d*, *a*, *b*, and ν are determined, for a large set of anesthetics, using the method of multiple linear regression analysis. The results obtained (Abraham et al. 1991; Davies et al. 1974) suggest that all the factors contained in the equation, i.e., hydrophobicity, dipolarity, polarizability, and hydrogen-bonding, contribute to the overall interaction.

3.2 Weak Forces Stabilizing Structures of Biological Macromolecules

Biological macromolecules, the complex functional units of biochemical systems, are held together by several reversible and noncovalent interactions and associations. These play a pivotal role in the folding of proteins, the recognition of substrates, and the interactions between receptors and ligands. The weak forces responsible for the right structure and functioning of biological macromolecules consist of electrostatic interactions, van der Waals forces, hydrogen bonds, and hydrophobic interactions (ChemgaPedia 2006).

The process of the breaking and remaking of hydrogen bonds enables functional proteins to change from one conformation to another. For example, neurotransmitter substances, themselves capable of forming hydrogen bonds and interacting through van der Waals forces and hydrophobic forces, lead to conformational changes by breaking hydrogen bonds in proteins (Celie et al. 2004; Reeves et al. 2003). Not only neurotransmitter molecules but many anesthetic molecules are capable of interacting by such weak forces also, and they have therefore the potential of disrupting functions of biologically important macromolecules such as proteins.

As already suggested in the previous section when multiple linear regression analyses of thermodynamic parameters were discussed, different weak forces may combine and superimpose in anesthetic actions. For example, the functional effects of the binding of ligands such as the neurotransmitter acetylcholine or serotonin are thought to depend on the simultaneous interactions involving several hydrogen bonds, cation–π interactions, dispersion forces, and hydrophobic forces (Celie et al.

2004; Reeves et al. 2003; Thompson et al. 2005). The effect of a combination may well be more than just the sum of the different interaction energies and lead to synergistic effects. Therefore, even small contributions may become very important in a combination of different contributing forces. Depending on the proteins and neuronal networks involved for any particular effect of anesthesia, different combinations of these weak forces may become relevant.

3.3 Ion–Ion Interactions

Ion–ion interactions involve the strongest of the Coulombic electrostatic forces (ChemgaPedia 2006). Typical energies for ion–ion interactions at a distance of 0.5 nm are 250 kJ/mol (ChemgaPedia 2006). Many intravenous anesthetics can be ionized and are present, at neutral pH, both in their neutral and their charged forms. Clinical compounds that are partly ionized at neutral pH include, for example, the barbiturates, ketamine, etomidate, and the benzodiazepines. There are examples demonstrating distinct actions of charged intravenous anesthetics and their neutral counterparts (Kendig 1981; Frazier et al. 1975). While direct evidence for ion–ion interactions is yet lacking for general anesthetics, electrostatic repulsion between the charged form of lidocaine and a Na^+ ion in the selectivity filter has been suggested to occur in voltage-dependent sodium channels (Tikhonov et al. 2006).

3.4 Ion–Dipole Interactions

The strength of ion–dipole interactions is weaker than that of ion–ion interactions, and it decreases rapidly with distance (ChemgaPedia 2006). The typical energies for ion–dipole interactions at a distance of 0.5 nm are 15 kJ/mol (ChemgaPedia 2006). In biochemical processes this type of interaction plays an important role, e.g., during hydration, complex formation, and cation–π interactions. For the squid axon, it has been suggested that alcohols and anesthetics adsorb at the membrane interface, thereby changing its electric field and the membrane potential through their dipole moments (Haydon and Urban 1983). These changes are then postulated to impact on the gating mechanisms that involve the translocation of net charges (Hille 2001). In gramicidin A pores, it has been proposed that their electrical conductance, i.e., ion flow through them, is affected by dipole potentials generated by *n*-alkanols adsorbed at the membrane interface (Pope et al. 1982).

3.4.1 Hydration

When ions dissolve in water, the dipolar water molecules will be attracted to them and associate with them depending on their partial charges (ChemgaPedia 2006). The

water molecules form several layers (hydration shells), the first layer depending primarily on ion–dipole interactions, and further layers being held together by hydrogen bonds. The number of coordinating water molecules depends on the size of the ion and its charge. The hydration shells of ions effectively increase the ionic radius, thereby influencing their diffusion through pores and ion channels (Hille 2001). The selectivity filters of ion channel proteins contain such ions that are in contact with water (Hille 2001). In addition, most biological macromolecules carry negative charges and are surrounded by their own hydration shells that help in stabilizing their conformations. The water molecules in these hydration shells are much more ordered and structured than they are in bulk water. Anesthetics can through the process of clathrate formation interfere with the structure of water in these hydration shells, as was first observed by Pauling (1961) and Miller et al. (1961) independently.

3.4.2 Cation–π Interactions

Cation–π interactions are strong electrostatic interactions that occur between a π-electron cloud and an atom that carries a full or partial positive charge (ChemgaPedia 2006). Cations involved are mainly metal ions or partially positively charged side chains that interact with the aromatic side chains of phenylalanine, tyrosine, or tryptophane (Fig. 2). Thus these positive charges can interact with the surfaces of nonpolar, aromatic structures. As a first approximation, these interactions arise from electrostatic attraction between the positive charge of the cation and the quadrupole moment of the aromatic system. Studies to estimate the strength of such interactions suggest that it may contribute as much as several kilocalories per mole of energy to stabilize the binding of ligand to protein (Beene et al. 2002). Because binding affinity is related logarithmically to binding energy, cation–π interactions may enhance binding affinity by several orders of magnitude (Raines 2005).

Cation–π interactions have been recognized as an important noncovalent force in biochemical macromolecules, particularly in proteins. They have been identified in the function of acetylcholine receptor channels and 5-HT_3 receptor channels (Beene et al. 2002), generally as a component in ligand-receptor interactions and in

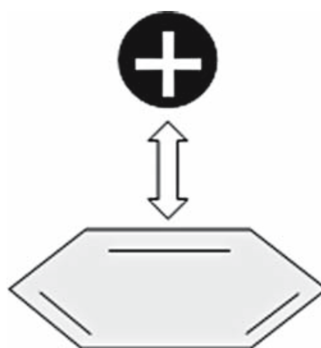

Fig. 2 Cation–π interaction: strong electrostatic interactions between a π-electron cloud of an aromatic ring and an atom that carries a full or partial positive charge

the stabilization of α-helices, in the binding reaction between proteins and DNA, and for the permeation of metal ions through ion channels. Thus by virtue of their π-electron clouds, aromatic anesthetics may engage in attractive electrostatic interactions with cationic atomic charges on protein targets. For example, volatile aromatic drugs inhibit *N*-methyl-d-aspartate (NMDA) receptor-mediated currents with potencies that are highly correlated with their abilities to engage in cation–π interactions (Raines 2005).

3.5 Van der Waals Interactions (Dipole–Dipole)

Often the term "van der Waals interaction" is loosely used as a synonym for weak intermolecular forces (ChemgaPedia 2006). In the narrower sense it describes intermolecular forces with attractive interaction energies that decrease with the sixth power of distance, because they arise from dipole–dipole interactions (ChemgaPedia 2006). These interactions occur between all kinds of atoms and molecules, even when those are nonpolar. Van de Waals forces can be attractive and repulsive, attraction dominating for larger distances between the interacting parts. Typical energies for dipole–dipole interactions at a distance of 0.5 nm are 0.3–2 kJ/mol (ChemgaPedia 2006). Thus van der Waals forces are quite weak, but they are additive. Their strengths grow with increasing sizes and polarizabilities of the molecules involved. When contact becomes too close, there will be strong repulsion caused by positively charged nuclei as well as by fully occupied orbitals (Pauli exclusion principle). The attractive and repulsive forces of van der Waals interactions are described mathematically by the Lennard-Jones potential.

Van der Waals interactions become particularly important in biological systems when two molecules consisting of many atoms approach each other. The interaction between ligand and receptor is primarily of electrostatic origin. Electrostatic forces govern the approach and the alignment of the ligand toward the protein. The probability that a sizable number of atoms of a ligand have by chance just the right distance to the atoms of the binding regions is very low. Thus the high selectivity and stereospecificity of ligand and protein interactions arises quite substantially from van der Waals interactions (ChemgaPedia 2006). Three components of van der Waals interactions are distinguished and described in the following: permanent dipole–permanent dipole, permanent dipole–induced dipole, and fluctuating dipole–induced dipole.

3.5.1 Permanent Dipole–Permanent Dipole

Of the three kinds of dipole interactions those between permanent dipoles (Fig. 3) are the strongest (ChemgaPedia 2006). There are many anesthetics that possess a permanent dipole moment, including the halogenated ethers and alkanes, while cyclopropane and xenon have none. The dipole moment of sevoflurane (3.3 debye) is quite similar in magnitude to that of a peptide bond (3.7 debye). Therefore, apart

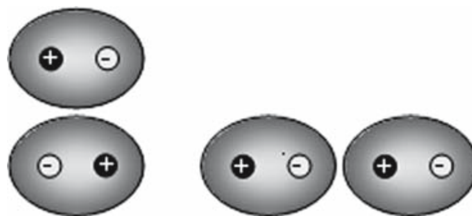

Fig. 3 Permanent dipole–permanent dipole interaction: dipoles can associate either head to tail or in an antiparallel orientation

from interactions with side chains of amino acids, anesthetics carrying permanent dipole moments may interact with proteins in several ways at many positions. Binding of anesthetics to human serum albumin has been suggested to involve permanent dipole interactions (Eckenhoff 1998).

3.5.2 Permanent Dipole–Induced Dipole (Induction Effect)

Dipole interactions may be observed between a dipole and a nonpolar molecule if the latter is polarizable (ChemgaPedia 2006). Polarizability arises if the electron cloud of an atom is distorted in the presence of a strong dipole moment (Fig. 4). For example, as noble gases are polarizable, a permanent dipole will be able to induce a dipole in them, giving rise to electrostatic Debye forces between the permanent and the induced dipole. Thus even the inert gas and anesthetic xenon are capable of interacting with proteins.

Polarizability increases with atomic size, and thus should become more prominent in those inhalation anesthetics that contain larger halogens such as chlorine or bromine. Eckenhoff and Johansson (1997) have observed that, for a given structure, both anesthetic potency and degree of metabolism are progressively increased as heavier halogens are substituted (Harris et al. 1992; Targ et al. 1989), suggesting that this type of van der Waals force may be important in producing anesthetic binding interactions in some relevant target.

3.5.3 Fluctuating Dipole–Induced Dipole (Dispersion or London Forces)

Dispersion forces, also called London forces, arise from spontaneous fluctuations of electron densities within atoms and molecules (ChemgaPedia 2006). The constant motion of the electrons in the molecule causes rapidly fluctuating dipoles even in the most symmetrical molecule such as monatomic molecules and noble gases. These fluctuations give rise to the formation of temporary electric dipoles that, in turn, will induce further dipoles in adjacent molecules (Fig. 5). Dispersion forces may act between completely apolar molecules. They are the weakest of all dipole–dipole interactions.

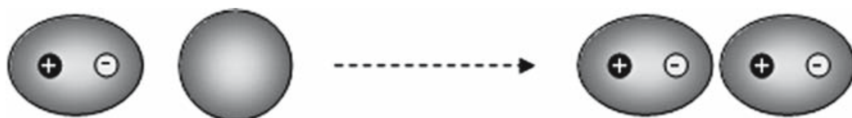

Fig. 4 Permanent dipole–induced dipole interaction: a strong permanent dipole can induce a temporary dipole in a polarizable nonpolar molecule

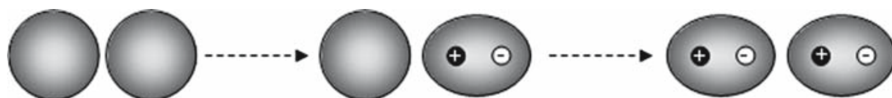

Fig. 5 Fluctuating dipole–induced dipole interaction: spontaneous fluctuations of electron densities within a symmetrical, apolar molecule create dipoles that, in turn, can induce dipoles in another polarizable, nonpolar molecule

The ease with which the electrons of a molecule, atom or ion are displaced by a neighboring charge is called polarizability. Anesthetic molecules are polarizable, even noble gases such as helium or the clinical anesthetic xenon. Thus, contrary to what their names suggest, they are not completely inert. The more electrons there are, and the larger the distance over which they can move, the bigger the possible temporary dipoles and therefore the bigger the dispersion forces. This is why bigger molecules can interact more strongly and why the boiling points of the noble gases increase from helium (−269°C) to xenon (−108°C).

A special case of London forces are π–π interactions between aromatic rings (ChemgaPedia 2006). They are stronger than ordinary London forces because the charges are more mobile in conjugated π-systems. These aromatic interactions occur either as π-stacking or as face-to-face interactions (Fig. 6). π–π interactions are particularly responsible for shaping the tertiary structure of proteins with aromatic side chains. Propofol, etomidate, ketamine, benzodiazepines (such as midazolam), droperidol, morphine, and its fentanyl derivatives are but some examples of intravenous anesthetic compounds containing aromatic rings.

3.6 Hydrogen Bonding

Interactions of the form *D*-H···|*A* between a proton donor *D*-H and a proton acceptor |*A* are called hydrogen bonds (ChemgaPedia 2006). *D* and *A* are generally strongly electronegative atoms such as F, O, and N. The most common hydrogen bonds are formed between oxygen and nitrogen atoms, which can act both as proton acceptors and as proton donors due to their free electron pairs (Fig. 7). In many hydrogen bonds the distance between the atoms *A* and *D* is shorter than the sum of the van der Waals radii. Hydrogen bonds are directional and strongest when all three atoms involved in the bond are on a straight line. The interaction energy of

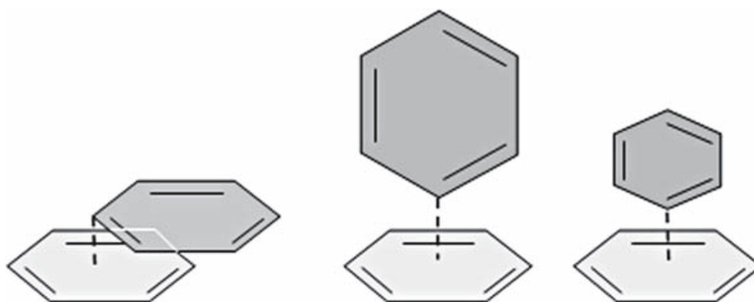

Fig. 6 π–π interactions between aromatic rings: a special case of London forces but stronger, these occur either as π-stacking or as face-to-face interactions

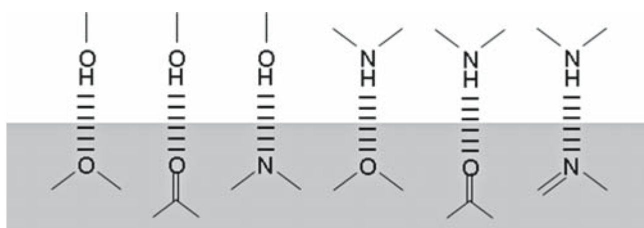

Fig. 7 Hydrogen bonds: the most common hydrogen bonds are formed between oxygen and nitrogen atoms, which can act either as proton acceptors (*shaded area*) or as proton donors (*not shaded area*) due to their free electron pairs

hydrogen bonds consists both of electrostatic contributions (dipole–dipole and dipole–ion interactions) and covalent contributions (three-center four-electron bonds). Energies for hydrogen bonds *D*-H···*A* range between 1 and 50 kJ/mol, energies between 10 and 50 kJ/mol are typical. In low barrier hydrogen bonds (F-H···F, O-H···O^-) the hydrogen atom is evenly spaced between the donor and the acceptor. This bridge is symmetrical with an angle of 180°, F-H···F being the strongest of all hydrogen bonds. All other hydrogen bonds are high-barrier bonds. Of these, O-H···O, O-H···N, N-H···O are the strongest. N-H···N form weaker hydrogen bonds, and the weakest are between O-H and π-electrons.

Infrared spectroscopists have known for more than half a century that aromatic rings (*Ar* in the following) can serve as acceptors for weak hydrogen bonds, with typical interaction energies of 4–8 kJ/mol (Sandorfy 2004). Indeed, amino acids like tryptophan, tyrosine, and phenylalanine possess aromatic rings. The existence of N-H···*Ar*, or OH···*Ar* hydrogen bonds in proteins was successfully demonstrated (Sandorfy 2004). These H-bonds are thought to play a pivotal role in determining the conformations and motions of proteins (Sandorfy 2004). They could be targets for a number of intravenous anesthetics that also possess aromatic rings. For example, the effect of aromatic amino acid

side-chain structure on halothane binding to four-helix bundles has been studied in detail (Johansson and Manderson 2002).

Hydrogen bonds are formed between single molecules (intermolecular) or within a molecule (intramolecular); they are the most important inter- and intramolecular interactions of all of biochemistry. Protein function depends on transitions between different conformations, which involve the breaking of old and remaking of new hydrogen bonds. Any substance such as anesthetics that can compete for hydrogen bonds would be disruptive to protein function. Polar interactions and the breakage of hydrogen bonds appear to be important factors for halogenated hydrocarbons containing an acidic hydrogen (Abraham et al. 1971; Davies et al. 1976; Urban and Haydon 1987), including the clinical anesthetics isoflurane, enflurane, sevoflurane, desflurane, halothane, and the obsolete clinical anesthetic chloroform. Hydrogen bonds may even be broken by substances that by themselves do not form hydrogen bonds as has been suggested for the interaction of *n*-alkanes with gramicidin A (Hendry et al. 1978; Elliott et al. 1983).

3.7 Hydrophobic Interactions

Hydrophobic interactions are weak interactions resulting from the tendency of hydrophobic molecules or hydrophobic portions of macromolecules to avoid contact with water (ChemgaPedia 2006; Tanford 1980; Tanford 1997). Hydrophobic forces are responsible for generating lipid bilayers that form the backbone of biological membranes. In aqueous solutions, water molecules close to hydrophobic interfaces are arranged such that their hydrogen bonds point away from the hydrophobic areas. This reduces the mobility of water molecules, leading to a breakdown of the free cluster structure of water. Because water molecules adjacent to a hydrophobic interface are highly ordered, they exist in a thermodynamically unstable state, favoring self-aggregation and minimization of the hydrophobic interfaces (Fig. 8). Thus hydrophobic interactions do not result from the van der Waals attraction of hydrophobic moieties but rather from the exclusion of water molecules from areas between hydrophobic interfaces (Fig. 8), resulting in a gain of entropy within the system. In contrast to hydrogen bonds, hydrophobic interactions are not directional.

The hydrophobic effect contributes significantly to the binding energies of ligands, for example, in 5-HT_3 receptors (Thompson et al. 2005) or nicotinic acetylcholine receptors (Schapira et al. 2002). Hydrophobic interactions are also important for the stabilization of peptide conformations by aliphatic and aromatic side chains (Lins and Brasseur 1995; Kauzmann 1959; Tanford 1997). In processes such as the hydrophobic collapse it plays an important role in protein folding. The contribution is proportional to the surface of the hydrophobic moieties involved. The observation that the Meyer-Overton rule holds in so many interactions between proteins and anesthetics (Urban et al. 2006) underscores the importance of hydrophobic effects in anesthetic action.

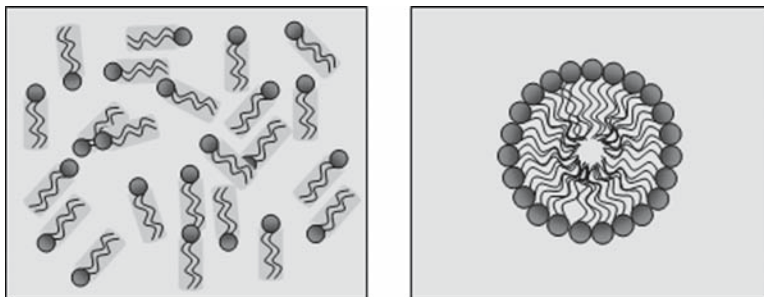

Fig. 8 Hydrophobic interactions: water molecules adjacent to a hydrophobic interface (*shaded areas*) are highly ordered, thus in a thermodynamically unstable state. Self-aggregation minimizes the hydrophobic interfaces

4 Molecular Sites of Anesthetic Action

4.1 Introduction

Following the previous sections' review of anesthetic targets and the various interactions that anesthetics can undergo, we shall finally consider molecular sites of anesthetic action that have been identified. This subject has been reviewed extensively, so only a selection of references is given here (Evers and Crowder 2005; Franks 2006; Koblin 2005; Urban et al. 1997; Sonner et al. 1950; Rudolph and Antkowiak 2004; Richards 1980; Miller 1985; Little 1996; Campagna et al. 1954; Urban 2002; Urban and Bleckwenn 2002; Overton 1901; Seeman 1972). Unfortunately, in the end, most investigations still represent black box approaches, despite the fact that the molecular configuration of the investigated anesthetics and related drugs can be varied systematically and although the molecular structure of the target sites can be altered methodically through site-directed mutagenesis. However, the spatial and temporal resolution needed for visualizing directly anesthetic action on molecular structures at the atomic scale is mostly beyond anything that is technically feasible today; the resolution of the static ion channel structure is currently limited to about 0.2 nm (Valiyaveetil et al. 2006; Unwin 2005). Therefore, for any one particular interaction it is in most cases impossible to be certain of which and how many molecular structures a drug is contacting, which conformational changes are triggered, and whether amino acid substitutions have altered the secondary and tertiary structures of proteins even before drugs interact with them.

The simpler the molecular constitution of an anesthetic-related drug, the more likely it is that it interacts not only with several molecular sites within a biological macromolecule but also with a whole range of different biological macromolecules. Thus the functional endpoint determined in experiments will only in very rare circumstances result from just a single molecular interaction but rather from an integration over time and space of several and dynamic molecular actions. Even

seemingly small molecular changes in either drug constitution or target site structure may thus not be attributable to a change in just a single molecular force or site. Unless time-resolved visualization in the 0.1-nm range and below can be achieved for interactions between drugs and their molecular targets, their identification will remain indirect, depending on the observation of function instead.

4.2 Lipid Bilayers

Lipid bilayers consisting of a bimolecular leaflet of lipids are the backbone of biological membranes. In the early 1960s it became possible to form artificial lipid bilayers. Their physicochemical properties were systematically characterized (Tosteson 1969; Haydon and Hladky 1972) and it was discovered that anesthetics have many actions on lipid bilayers (Koblin 2005; Miller 1985; Seeman 1972). Purely hydrophobic anesthetics were found to be located preferentially in the lipid membrane hydrocarbon core, while amphipathic molecules tended to be localized predominantly in the membrane interface (North and Cafiso 1997; Tang et al. 1997; Pohorille et al. 1996). Purely hydrophobic anesthetics increase membrane thickness and raise their surface tension (Haydon et al. 1977). Lateral pressure profiles in membranes are also changed (Cantor 1997). The insertion of anesthetic molecules into lipid membranes causes them to become more fluid and disordered (Firestone et al. 1994). The increase in lipid fluidity resulting from the absorption of inhaled agents can vary considerably (Ueda et al. 1986) and depends on the lipid system examined, the position within the membrane, and the method of fluidity measurement (Baber et al. 1995; North and Cafiso 1997; Tsuchiya 2001; Vanderkooi et al. 1977). Phase transition temperatures of bilayer membranes may decrease (Galla and Trudell 1980; Tsuchiya 2001; Vanderkooi et al. 1977). Lateral phase separation may result (Trudell 1977). Anesthetics may also change membrane electrical properties such as membrane dielectric constant (Enders 1990) or surface dipole potentials (Reyes and Latorre 1979). Inhaled agents have been reported to increase the ion permeability of liposomes in a concentration-related manner (Andoh et al. 1997; Barchfeld and Deamer 1985; Miller et al. 1972).

4.3 Protein Binding Sites

While there are many studies showing effects of anesthetics on protein function, in general they often fail to prove that anesthetics first bind to the proteins involved before they bring about the observed effects (Eckenhoff and Johansson 1997). Even in reconstituted lipid bilayer systems, for example, consisting only of highly purified sodium channels and no more than two different kinds of lipid molecules (Wartenberg et al. 1994), it is difficult to prove that the observed functional effects of anesthetics are only due to anesthetic binding to the protein. Indeed, it could be

shown that lipid bilayer composition modulated some functional anesthetic effects on purified sodium channels (Rehberg et al. 1995). Thus caution is advised when making inferences about binding based on functional studies.

Nuclear magnetic resonance (NMR) spectroscopy and photoaffinity labeling have been used as more direct approaches to study anesthetic binding to proteins (Evers and Crowder 2005). ^{19}F-NMR spectroscopic studies showed that isoflurane binds to approximately three saturable binding sites on bovine serum albumin, a fatty acid-binding protein (Dubois and Evers 1992). These results were confirmed by Eckenhoff and colleagues when they used ^{14}C-labeled halothane to photoaffinity label anesthetic binding sites on bovine serum albumin (Eckenhoff and Shuman 1993). They were able to identify the specific amino acids that were photoaffinity labeled by [^{14}C]halothane.

This binding was eliminated by co-incubation with oleic acid, consistent with the assumption that isoflurane binds to the fatty acid-binding sites on albumin. Other clinical anesthetics, such as halothane and sevoflurane, competed with isoflurane for binding to bovine serum albumin (Dubois et al. 1993). These studies provide suggestive evidence that at least certain anesthetics can compete for binding to the same site on a protein.

Currently, NMR and photoaffinity labeling techniques can only be applied to purified proteins available in relatively large quantities. The muscle-type nicotinic acetylcholine receptor is one of the few membrane proteins that has been purified in large quantities. It could be shown that halothane binds to this protein, but the pattern of photoaffinity labeling is complex, indicative of multiple binding sites (Eckenhoff 1996). Binding to specific sites on the nicotinic acetylcholine receptor could also be shown with a new and different technique involving 3-diazirinyloctanol. Most recently, Miller and colleagues have developed a general anesthetic that is an analog of octanol and functions as a photoaffinity label (Husain et al. 1999).

Other approaches to identify the location and structure of anesthetic binding sites have involved site-directed mutagenesis of candidate anesthetic targets in combination with molecular modeling. Using this strategy the location and structure of the alcohol binding site on γ-aminobutyric acid $(GABA)_A$ and glycine receptors has been predicted (Wick et al. 1998). An additional approach involves the use of model proteins such as gramicidin A (Hendry et al. 1978; Tang and Xu 2002; Pope et al. 1982) or four α-helix bundles with a hydrophobic core that can bind volatile anesthetics (Johansson et al. 1998).

4.4 Hydrophobic Pockets (Cavities) in Proteins

Considerable attention has been focused on preformed cavities within proteins as binding sites for inhaled anesthetics. Hydrophobic cavities within proteins are apparently quite common in proteins (Eckenhoff 2001). When proteins fold into complex structures, packing defects known as “cavities” are generated. These cavities are thought to introduce the necessary instabilities that facilitate

conformational changes accompanying protein function (Eckenhoff 2001). The size of some of these cavities permits the occupation by anesthetic molecules. A recent screen of the Protein Data Bank for potential targets of halothane identified 394,766 total cavities, of which 58,681 cavities satisfied the fit criteria for halothane (Byrem et al. 2006). Experimental data support the hypothesis that small molecules can bind in cavities formed between α-helices in proteins (Trudell and Bertaccini 2002).

X-ray diffraction crystallography has been used to reveal details of the three-dimensional structure of anesthetic sites that NMR and photoaffinity techniques cannot provide (Evers and Crowder 2005). Because X-ray diffraction requires crystallized membrane proteins, it has so far only been used for a small number of proteins. One of the first studies of this type was performed with myoglobin. It was shown that the anesthetic molecules xenon and cyclopropane were able to bind in the hydrophobic core of a protein and that the size of the hydrophobic binding pocket could account for a cutoff in the size of anesthetic molecules that can bind in that cavity (Schoenborn et al. 1965; Schoenborn 1967).

Another example of a hydrophobic pocket has been demonstrated with X-ray diffraction for halothane binding deep within the enzyme adenylate kinase (Sachsenheimer et al. 1977). The halothane binding site was identified as the binding site for the adenine moiety of adenosine monophosphate, a substrate for adenylate kinase. Another example of anesthetics binding to endogenous ligand binding sites is provided by firefly luciferase, where two molecules of the anesthetic bromoform bind in the luciferin pocket, one of them competitively with luciferin and the other one noncompetitively (Franks et al. 1998). Human serum albumin has also been successfully crystallized and the X-ray crystallographic data show binding of propofol as well as of halothane to preformed pockets that had been shown previously to bind fatty acids (Bhattacharya et al. 2000).

The binding energies of anesthetics to these sites of action appear to be small, so that these molecules bind presumably adventitiously to preexisting cavities or sites. Consequently, the binding event is not thought to cause an “induced fit” in a protein site or even provide substantial reorganization of an internal cavity (Harris et al. 2002). Anesthetic binding to these cavities affects protein stability depending on their native sizes: proteins having intermediate pre-existing cavities are destabilized, presumably resulting from preferential binding of the anesthetic to less stable intermediates with enlarged cavities. Proteins containing larger cavities are stabilized by the anesthetic, indicative of binding to the native state (Miller 2002; Eckenhoff 2002).

The volume of the cavity or binding pocket constitutes a constraint on the anesthetic molecules that may bind. This volume may depend on the conformation of the protein. This has been shown for glycine receptor channels, possessing binding pockets with volumes that are different in the resting (smaller) and in the activated (larger) state (Harris et al. 2002). The volume of the cavity and proposed anesthetic binding site in $GABA_A$ receptor channels is estimated to range between 0.25 and 0.37 nm^3, quite likely constituting a common site of action for the anesthetics isoflurane, halothane, and chloroform (Jenkins et al. 2001). Modulation of human

5-HT_{3A}-mediated currents by volatile anesthetics exhibits a dependence on molecular volume similar to *n*-alcohols, suggesting that both classes of agents may enhance 5-HT_{3A} receptor function via the same mechanism (Stevens et al. 2005). The data suggest an apparent size of 0.120 nm^3 for the cavity (Stevens et al. 2005), which modulates anesthetic and *n*-alcohol enhancement of agonist action on the 5-HT_{3A} receptor.

These and other studies have demonstrated that possible sites of anesthetic action exist within the transmembrane subunits of the superfamily of ligand-gated ion channels. The exact molecular arrangement of this transmembrane region remains at intermediate resolution with current experimental techniques (Eckenhoff 2001). In order to produce a more exact model of this region, homology modeling methods combined with experimental data have been used. This approach produced a final structure possessing a cavity within the core of a four-helix bundle. Converging on and lining this cavity are residues known to be involved in modulating anesthetic potency. Thus cavities formed within the core of transmembrane four-helix bundles may be important binding sites for volatile anesthetics in the ligand-gated ion channels (Bertaccini et al. 2005).

4.5 Hydrophilic Crevices in Proteins

Water-filled crevices in proteins, apart from hydrophobic cavities, have also been implicated as molecular sites of anesthetic action. Akabas et al. (2002) suggest that crevices and cavities form in the membrane-spanning domains during $GABA_A$ receptor gating. Since a vacuum is energetically unfavorable, water moves in, thereby facilitating conformational change. These water-filled crevices extend from the extracellular surface into the interior of the $GABA_A$ receptor protein. Anesthetics, by preferentially filling these crevices/cavities, could stabilize receptor conformations other than the resting state, altering the probability of channel opening (Akabas et al. 2002). While this site is still quite hypothetical at present, it considers the possibility that anesthetics may enter proteins by transfer to an annular ring formed by the four-component interface of the ligand-binding and transmembrane domains of the protein, the phospholipid bilayer, and the interfacial water layer. This route that anesthetics may take constitutes an alternative to diffusion down the water-filled lumen of the ion channel or dissolution in the phospholipid bilayer followed by transfer through the lipid–protein interface of the ion channel (Trudell and Bertaccini 2002).

4.6 Lipid/Protein Interfaces

Integral membrane proteins are essential for mediating numerous physiological functions. In order to function successfully, membrane proteins must perform

properly within, and at the same time interact with, the lipid membrane in which they undergo conformational changes while carrying out their complex functions. There is much evidence for a strong effect of the properties of lipid bilayers on the function of membrane proteins (Trudell and Bertaccini 2002; Rebecchi and Pentyala 2002).

Reconstitution studies have provided the best evidence that the lipid environment may significantly affect the properties of integral membrane proteins. In reconstitution studies it is actually possible to reinsert proteins, which have been removed from their native membranes, into artificial lipid bilayer membranes of defined lipid composition. A number of diverse reconstituted proteins have been found to have altered functions, depending on the composition of the surrounding lipids (Zakim 1986). Specific properties of phospholipids, such as head group composition, and general properties of the hydrophobic bilayer, such as micro viscosity, can have dramatic effects on protein function. This leads to the expectation that if the properties of lipid bilayers have been changed by anesthetics in a comparable way, then protein function should also be altered.

Nash (2002) takes issue with the fact that lipid targets of anesthetic action have fallen from favor. He argues that he knows of no decisive experiment that eliminates lipid targets from contention, particularly if one acknowledges the possibility that subtle alterations of bilayers by volatiles anesthetics might impact on the function of proteins imbedded in them. The function of the ion channel-forming polypeptide gramicidin A is modulated by the lipid environment (Hendry et al. 1978; Pope et al. 1982). Anesthetic changes of membrane parameters have been postulated to directly affect sodium channel and potassium channel function in the squid giant axon (Urban 1993). The lipid environment alters the actions of pentobarbital on purified sodium channels reconstituted in planar lipid bilayers (Rehberg et al. 1995). Studies using site-directed mutations in ligand-gated ion channels combined with molecular modeling suggest that a primary point of action of anesthetics is in the transmembrane domain of these channels (Trudell and Bertaccini 2002). Another example involves certain protein kinases where anesthetics might operate at the protein/lipid interface by changing the lateral pressure profile (Rebecchi and Pentyala 2002).

4.7 Protein/Protein Interfaces

The possibility that anesthetics might be able to act at the interface between protein subunits or at the interface between different proteins has not been explored extensively. It has been suggested that anesthetics binding to such sites might disrupt, for example, allosteric transitions at domain/domain interfaces of protein kinases or prevent agonist-induced dissociation of receptor from the heterotrimeric G proteins (Rebecchi and Pentyala 2002).

4.8 Relevant Sites for Anesthetics

Figure 9 summarizes sites of anesthetic action that have been identified in lipid bilayers and in ion channels, the latter representing the best-studied class of membrane proteins in this context. Anesthetics may differ in the spectrum of interaction sites depending on their physicochemical properties and the structures of the biological macromolecules. Within the bilayer, anesthetics may act (1) at the interface between the lipid and the aqueous phase, (2) within the hydrophobic interior of the lipid bilayer itself (Urban et al. 1991; Trudell and Bertaccini 2002), or (3) between the lipid and membrane proteins. Anesthetics may bind to protein binding sites in contact with the aqueous phase, located either (4) inside the channel lumen of ion channels (Dilger 2002; Scholz 2002), or (5) at the water/protein interface. (6) Water-filled crevices or water channels inside or adjacent to membrane proteins have been implicated (Trudell and Bertaccini 2002). Anesthetics may bind (7) within the core of the membrane protein itself, between hydrophobic α-helices (Frenkel et al. 1990) and form hydrophobic or lipophilic pockets (Trudell and Bertaccini 2002). (8) Anesthetics may disturb interactions between subunits of a protein or between different proteins (Trudell and Bertaccini 2002; Rebecchi and Pentyala 2002). In addition, Sandorfy (2002) has pointed out that carbohydrates that are covalently attached to membrane proteins may also constitute sites of anesthetic actions.

Which of the molecular sites are relevant for clinical anesthesia? The answer to this question requires knowledge of the neuronal networks critical to general anesthesia or to one of its essential clinical components. When these relevant neuronal networks will have been identified, it should then become possible to assess which molecular sites contribute to clinical anesthesia.

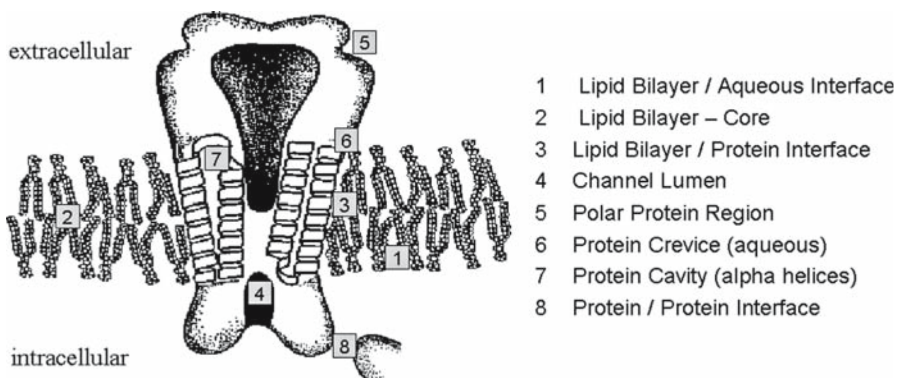

Fig. 9 Summary of identified molecular sites of anesthetic action in membranes and in embedded proteins

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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)*

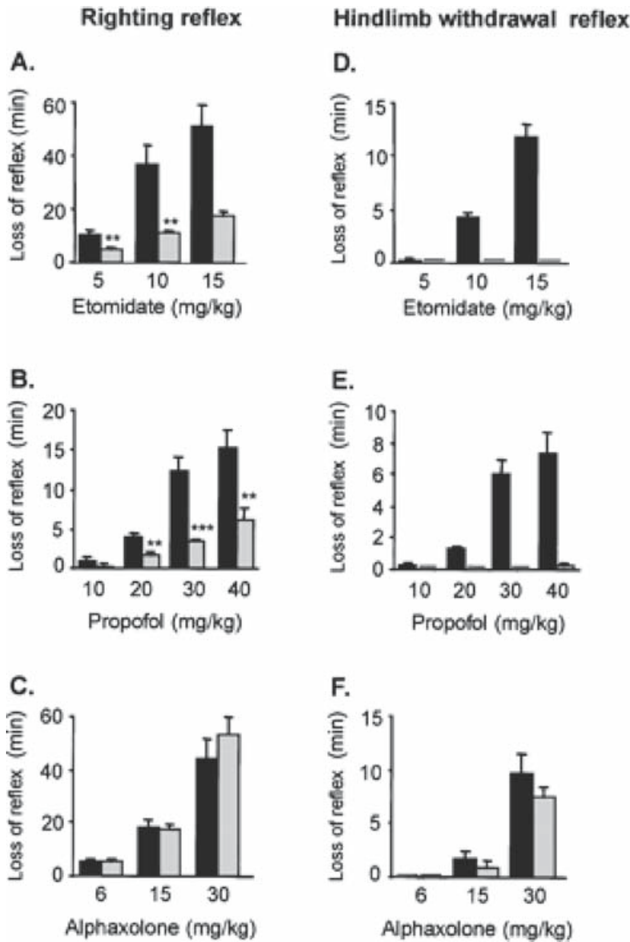

Fig. 1 Behavioral responses to i.v. anesthetics in wildtype and β_3*(N265M)* mice. Reduction in the duration (in minutes) of the loss of righting reflex (LORR) induced by **a** etomidate and **b** propofol in β_3*(N265M)* mice vs wildtype. Etomidate (15 mg/kg) and propofol (40 mg/kg) were lethal for 50% and 58% of the wildtype, respectively, but none of the β_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 β_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 β_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 β_3*(N265M)* and wildtype mice. All drugs were administered intravenously. Wildtype mice, *black shading*, β_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)