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Molecular targets underlying general anaesthesia

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The discovery of general anaesthesia, over 150 years ago, revolutionised medicine. The ability to render a patient unconscious and insensible to pain made modern surgery possible and general anaesthetics have become both indispensible as well as one of the most widely used class of drugs. Their extraordinary chemical diversity, ranging from simple chemically inert gases to complex barbiturates, has baffled pharmacologists, and ideas about how they might work have been equally diverse. Until relatively recently, thinking was dominated by the notion that anaesthetics acted 'nonspecifically' by dissolving in the lipid bilayer portions of nerve membranes. While this simple idea could account for the chemical diversity of general anaesthetics, it has proven to be false and it is now generally accepted that anaesthetics act by binding directly to sensitive target proteins. For certain intravenous anaesthetics, such as propofol and etomidate, the target has been identified as the $GABA_A$ receptor, with particular subunits playing a crucial role. For the less potent inhalational agents, the picture is less clear, although a relatively small number of targets have been identified as being the most likely candidates. In this review, I will describe the work that led up to the identification of the $GABA_A$ receptor as the key target for etomidate and propofol and contrast this with current progress that has been made in identifying the relevant targets for other anaesthetics, particularly the inhalational agents.
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Discovery of general anaesthesia

The discovery of general anaesthesia is a remarkable story rich with human tales of serendipity, impropriety, noble ambition and inflated egos. At its most attractive, it features a dedicated and modest country doctor, Crawford Long, who was clearly the first practitioner of modern general anaesthesia, and whose only failing was his timidity in making his work using ether in the early 1840s more generally known. A few years later, and in ignorance of Crawford Long, the Connecticut dentist Horace Wells hit upon the idea of using nitrous oxide while watching a public demonstration of its powers of intoxication (Figure 1a). Having satisfied himself by self-administration that it ameliorated the pain of tooth extraction, he conducted an abortive demonstration at the Massachusetts General Hospital, and was publicly humiliated by this failure. A former apprentice and colleague of Wells, another dentist called William Morton subsequently took up Wells' basic idea of a gaseous anaesthetic agent, together with the suggestion of chemist and physician Charles Jackson to use ether, a much more potent drug, and this culminated in the widely known public demonstration of ether anaesthesia on 16 October 1846 (Figure 1b). There followed several years of unedifying wrangling between Jackson, Morton and Wells as to who deserved credit for the discovery of general anaesthesia, with Wells eventually committing suicide, Jackson dying in an insane asylum and Morton dying penniless of a heart attack at the age of 48. A sad backdrop to one of the most momentous discoveries of modern pharmacology.

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Definition of general anaesthesia

Before describing the development of ideas about how general anaesthetics act, it is worth considering the state itself and how it might best be defined. Considerable and unnecessary confusion has been introduced when considering this question, often by practising anaesthetists who for perfectly good reasons are concerned with defining the most appropriate clinical state for their patients. Hence, discussions about anaesthesia ideally providing immobility, analgesia, amnesia, and muscle relaxation, for example, are transmuted into becoming a definition of a general anaesthetic. This is to confuse what is desirable clinically with what might be a logical and commonsense definition of a general anaesthetic. The drugs that we commonly class as general anaesthetics (Figure 2) vary greatly in their ability to cause analgesia, muscle relaxation and amnesia and really only have one defining feature in common – they induce a reversible loss of consciousness at low concentrations. They also cause an increasing lack of responsiveness at higher concentrations. Thus, a definition of general anaesthesia might simply be 'a reversible, drug-induced loss of consciousness', recognising that we will also need to specify a number of behavioural end points to characterise lack of responsiveness as the anaesthetic concentration increases.

For example, in humans, a loss of response to a verbal command occurs at about 100 μM for the inhalational anaesthetic isoflurane (in free aqueous solution) and at about 9 μM for the barbiturate thiopental. Essentially the same concentrations cause a loss of righting reflex (LORR) in rodents. For both humans and rodents, concentrations approximately three times higher are required to prevent a purposeful response to a painful stimulus (i.e. about 300 μM for isoflurane and 23 μM for thiopental). Three things are reflected in these data that are generally true. The anaesthetic EC_{50} concentration depends upon the end point and the anaesthetic, but not upon the animal species (at least for mammals). More specifically, loss of consciousness in humans (failure to respond to a verbal command) occurs at about the same concentration as LORR in rodents, and both occur at considerably lower concentrations than the loss of a purposeful response to a painful stimulus, although this ratio of concentrations does vary somewhat between different anaesthetics.

Finally, the temptation to refer to the loss of behavioural end points in animals as 'anaesthesia', or 'sedation' or 'hypnosis' has engendered considerable confusion and is probably best avoided, although admittedly this is often hard to resist as a useful shorthand.

In this review, I will focus specifically on which molecular targets are responsible for the principal actions of general anaesthetics and leave the question of where they act at an anatomical level for another day.

Figure 1 (a) A playbill from 1824 advertising an entertainment at the Savoy Theatre in London in which a demonstration of the 'exhilarating effects' of laughing gas (nitrous oxide) was promised to 'any of the audience who may chuse [sic] to inhale it'. It was at a similar occasion in Hartford Connecticut in December 1844 that Horace Wells had seen nitrous oxide demonstrated and where he observed that a volunteer, Samuel Cooley, a clerk at the local drugstore, had injured his leg without any apparent pain. Reproduced with permission from the Victoria and Albert Museum. (b) A re-enactment of the first public demonstration of the use of ether in surgery. This photograph, dated 1847 is a staging of the original event at the Massachusetts General Hospital, in October 1846 by some of the participants. William Morton is at the head of the patient holding his flask of ether. The surgeon, John Collins Warren, is facing the camera with his hands on the patient's leg. The photograph is a daguerreotype by Albert Sands Southworth and Josiah Johnson Hawes and is in the J. Paul Getty Museum, Los Angeles, © The J. Paul Getty Museum.

Early ideas about mechanisms

Ideas as to how general anaesthetics might work followed soon after their introduction into clinical practice. An early suggestion, made within a year of the public demonstration in Boston, was that anaesthetics might actually be extracting fatty substances from the brain. Happily, this idea proved to be false. Nonetheless, the first theory that wielded a major influence also involved lipids. It was put forward by Meyer and Overton, who had independently observed that the potency of an anaesthetic increased in direct proportion to

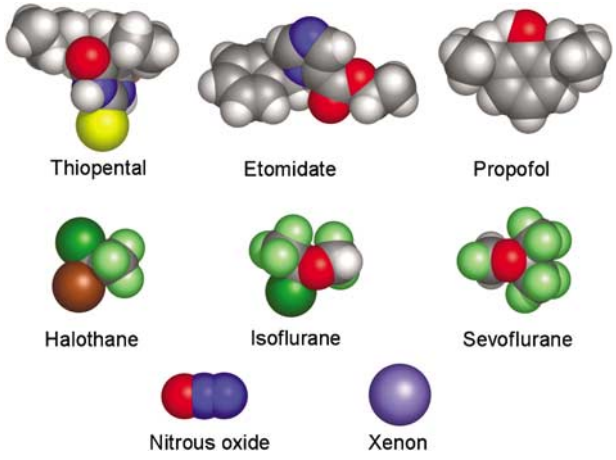

Figure 2 A selection of general anaesthetics that are used clinically shown as space-filling models. The top row are intravenous anaesthetics, the middle row are volatile inhalational agents and the bottom row are inorganic gases. The older agents are on the left and the most recently introduced are on the right. (A distinction is sometimes made between 'vapours' such as isoflurane and 'gases' such as nitrous oxide. However, the physical states of a vapour and a gas are indistinguishable; strictly speaking, a vapour only exists lower than a 'critical temperature', below which a high enough pressure can condense it into a liquid. The critical temperature of nitrous oxide, for example, is 36.4°C, which strictly makes it a vapour at room temperature, but a gas at body temperature.) The molecular models were drawn using PyMol (DeLano Scientific, San Carlos, CA, U.S.A.).

its oil/water partition coefficient. Once the role that lipids played in membrane structure became understood, this striking correlation was interpreted as meaning that the site of action of general anaesthetics was the lipid bilayer portion of nerve membranes (Figure 3a). The fact that the correlation had a unit slope made a simple molecular interpretation possible – during general anaesthesia, the concentration of all anaesthetics within the bilayer would be the same; so they must simply be acting by disrupting the normal state of the bilayer, with all anaesthetics being equally potent at this 'site'. What then followed were many years of speculation as to what form this disruption might take.

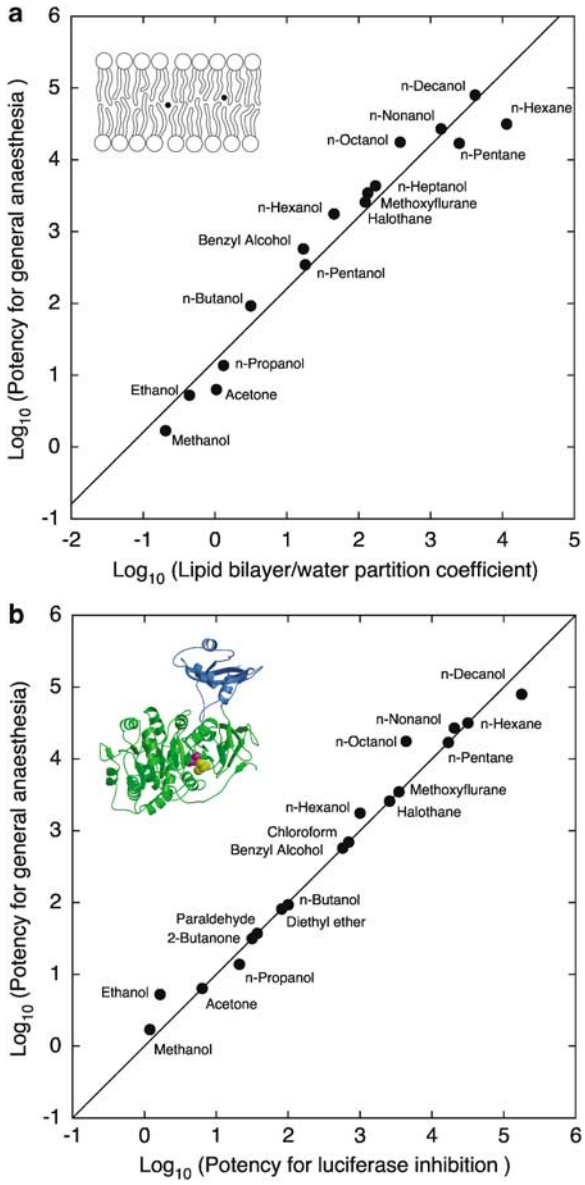

Figure 3 General anaesthetics act by binding directly to proteins. (a) The famous Meyer/Overton correlation has traditionally been interpreted as meaning that the primary target sites are the lipid portions of nerve membranes. In its modern form, shown here, a good correlation is seen to exist between the potency of an anaesthetic (≡reciprocal of its molar EC50 concentration for anaesthesia) and its lipid/water partition coefficient. (b) General anaesthetic potencies in animals can be correlated equally well with their ability to inhibit the activity of certain soluble enzymes, such as firefly luciferase, whose crystal structure is shown in the inset (Franks & Lieb, 1994). Reprinted with permission from Nature.

Refinement of lipid hypotheses and their shortcomings

In 1982, when my colleague Bill Lieb and I reviewed work on molecular mechanisms (Franks & Lieb, 1982), thinking was dominated by lipid theories of one sort or another. Broadly, they fell into four different camps. Anaesthetics were said either to increase lipid fluidity, trigger lipid phase transitions, change lipid bilayer dimensions or to alter bilayer permeability. All of these ideas had serious problems, some quantitative, some qualitative. The major quantitative problem is easy to state – at anything like a pharmacologically relevant concentration, the proposed changes in the bilayer were implausibly small; for example, they could usually be mimicked by a very small rise in temperature. The reason why so many theories could be criticised on this basis had to do with a lack of attention to which anaesthetic concentrations are pharmacologically relevant. Unfortunately, this problem persists and I shall return to this point later.

Aside from quantitative problems, there were also significant qualitative difficulties with most of the lipid hypotheses. For example, they generally predicted that anaesthetic potency would increase with increasing temperature, because temperature mimicked the proposed effects of anaesthetics; yet, the reverse is generally true. They also, almost invariably, ignored the well-established 'cutoff effect', which was the observation that, above a certain point in an homologous series, anaesthetic potency abruptly disappears. Finally, although this line of argument emerged some years later, lipid theories have major difficulties in accounting for the fact that anaesthetic enantiomers usually display different anaesthetic potencies in animals (see later).

Perhaps the most serious problem of all with lipid theories was the fact that it was rarely, if ever, explained how the proposed perturbation in the lipid bilayer would then result in a dysfunctional membrane protein, which almost all theories agreed had to be the ultimate result. This crucial step in the chain of events was always rather along the lines of 'and then a miracle happens'. To be fair, the permeability theories held that lipids alone were the mediator, and permeability changes led to compromised neurotransmitter uptake. Like other lipid theories, however, this idea has not stood the test of time.

Why were lipid theories so popular? The main reason, of course, was the fact that they accounted for the observations of Meyer and Overton, leaving aside the various exceptions mentioned above. They also had a beguiling simplicity. But they also promised a solution to an intriguing observation that had transfixed the field for many years, but which is now largely consigned to history – pressure reversal. Since this is an historical review, I will spend a few moments reviewing this intriguing phenomenon.

The basic observation is that high pressure (of the order of 100 atm) appears to reverse general anaesthesia. This work originated with observations in the 1940s on the effects of pressure on luminescent bacteria, which was subsequently extended to aquatic animals, and eventually mammals. The fact that an anaesthetised mouse will regain its motor activity when exposed to 100 atm of helium does indeed sound like a

remarkable phenomenon. However, when one appreciates that the concentration–response curve for general anaesthesia is very steep, a relatively small change in anaesthetic potency is all that is needed to affect this dramatic 'reversal'. For mice, for example, the change in anaesthetic potency for a range of simple anaesthetic gases at 100 atm is only about 30%. The question then is whether pressure is causing a true pharmacological antagonism, by directly reversing the molecular effects of anaesthetics, or whether the effect is physiological, with pressure causing incidental changes in the animal, such as increasing the general level of excitability, which leads to a reduced anaesthetic potency.

As pressure, at constant temperature, can only act by reducing the volume, the simplest possible theory consistent with a direct pharmacological antagonism is one that supposes that anaesthetics act by simply increasing volume. This indeed was a popular theory (the critical volume hypothesis), first put forward by Lorin Mullins, but then quantitatively refined and strongly advocated by Brian Smith and Keith Miller and their colleagues. Although Bill Lieb and I pointed out (Franks & Lieb, 1982) that the data, at least for the simple gases, might equally well be explained in terms of displacing the anaesthetic from protein-binding sites, pressure reversal has never really been satisfactorily explained. The possibility that it is caused by a physiological antagonism remains a perfectly plausible explanation, and I recall Brian Smith conceding that an anaesthetised mouse restored to 'normality' by pressure 'didn't look like it would be too good at mental arithmetic'. Indeed, it is well known that pressure, in the absence of anaesthetics, causes an increased excitability and it might be that a generalised increase in excitable tone is sufficient to counteract the depressant effects of the anaesthetic. This would explain why pressure can reverse the actions of not just simple gases but also highly selective intravenous agents which bind to specific receptors with minimal changes in system volume, where a pharmacological antagonism is most difficult to imagine.

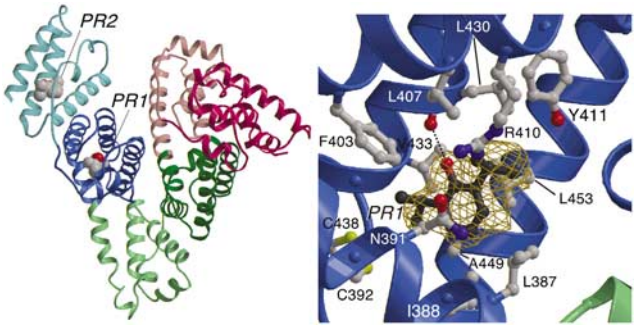

Figure 4 Propofol-binding sites on human serum albumin (Bhattacharya *et al*., 2000). Approximately 98% of propofol binds to blood constituents following administration. The major binding is to the carrier protein serum albumin. A crystallographic analysis shows two possible binding sites (PR1 in subdomain IIIA and PR2 in subdomain IIIB), but it is likely that only site PR1 is occupied under physiological conditions because of the strong binding of fatty acids to the PR2 site. The dashed line in the close-up view on the right represents a hydrogen bond. Propofol binds to a pre-formed cavity with very little perturbation of the local structure.

Work on model proteins

By the early 1980s, there were strong reasons for doubting the validity of lipid-based theories of general anaesthesia, but the evidence to support the obvious alternative, that anaesthetics acted by binding directly to protein targets, was lacking. However, work with a number of model proteins gave some encouragement (Franks & Lieb, 1982) and one class of proteins appeared to be particularly sensitive – the luciferase enzymes.

A good deal of work had been carried out on luminescent bacteria, but simple molecular interpretations in terms of protein or lipid targets were no easier than they were with whole animals. Work on cell-free systems was extremely limited, but what little there was suggested that the sensitivity of luciferase systems might have a simple interpretation at the molecular level. Subsequent work using the firefly luciferase enzyme showed (Franks & Lieb, 1984) that a wide variety of simple general anaesthetics could inhibit this enzyme by direct binding and there was an excellent correlation between the concentrations required for enzyme inhibition and those required for anaesthesia in animals (Figure 3b). The inhibition was competitive in nature and the data were consistent with all of the anaesthetics binding to a common site with a volume of about 250 ml mol^{-1}. The circumscribed dimensions of the binding pocket meant that the enzyme displayed a cutoff effect, and this provided a simple molecular interpretation of the cutoff effect observed with animals. The competitive nature of the inhibition, subsequently confirmed by crystallographic analysis, allowed these data to be readily extrapolatable to other proteins.

Crystallographic studies with other proteins (Figure 4) have reinforced the notion that anaesthetics act by binding to specific pockets or clefts on their protein targets, with probably only modest changes in local structure (Bhattacharya *et al*., 2000). However, crystallographic data on anaesthetics binding to ion channels are still some way off and these data will be required before this picture can be confirmed.

Anaesthetic concentrations

Before moving on to discuss the most likely targets in the central nervous system, I want to address the issue of appropriate anaesthetic concentrations to use for *in vitro* experiments which have, more than any other single factor, bedevilled this field. Anaesthetics are unusual in a number of respects, of which one is their relatively low therapeutic indices. For the inhalational agents, this is as low as 2–4. In other words, concentrations that are much higher than those used clinically are bound to be recruiting anaesthetic targets that are involved in unwanted side effects and many of these targets will not be important for anaesthesia itself, as defined above. With intravenous drugs it is obviously essential to take account of binding to blood proteins because this can greatly reduce the free aqueous concentration; in the case of propofol, protein binding reduces the effective concentration by a factor of nearly 50. It is also equally obvious that one cannot refer to the peak blood concentration following a bolus injection as the 'relevant' concentration for *in vitro* experiments, because pharmacokinetic factors greatly reduce the concentrations reaching the brain. However, for at least a few intravenous agents, quasi steady state concentrations required for various

end points have been determined and the corresponding free aqueous concentrations provide the appropriate benchmarks (Franks & Lieb, 1994).

A more subtle problem arises when dealing with volatile or gaseous anaesthetics, which invariably show a relatively large temperature dependence in their animal potencies, with potencies increasing as the temperature is lowered. This is an inevitable consequence of the anaesthetic molecule being transferred from the gas phase to their (condensed phase) targets, a physical effect which very largely accounts for changes in animal potencies. At low enough temperatures, hypothermia *per se* will also affect anaesthetic end points, but this is in addition to the inevitable increases in binding affinity with temperature. A simple practical solution to this problem is to express all anaesthetic concentrations as aqueous concentrations, which show a very much smaller temperature dependence (Franks & Lieb, 1994) than gas-phase concentrations.

All of the above problems have conspired together to result in hundreds of papers being published in which inappropriately high concentrations have been used. During the last decade or so, the situation has greatly improved and there is now a wide, although not universal, appreciation that, in this area of pharmacology in particular, concentrations matter.

Criteria for deciding which protein targets are relevant to general anaesthesia and which are not

There are a number of criteria, some more powerful than others, against which the relevance of a particular target can be judged. The first of these is plausibility. Is the protein expressed in appropriate anatomical locations to be relevant to the anaesthetic end point being studied (in so far as this judgement can be made), and are the effects of anaesthetics plausible in a qualitative sense? For example, if anaesthetics were to inhibit a receptor whose actions are known to be predominantly inhibitory, this would detract from its plausibility, while not, admittedly, ruling it out. Second, following the discussion above, the target protein should be significantly affected at pharmacologically relevant concentrations. But what is significant? There is no simple answer to this other than to say that targets that are very sensitive are, all other things being equal, more likely to be relevant than targets that are relatively insensitive. The third criterion takes advantage of the fact that many anaesthetics are chiral and their enantiomers usually show significantly different potencies in animals. If this difference in animal potency is reflected in the putative target, this adds greatly to its plausibility.

The last criterion is the most powerful of all, but the most difficult to utilise in practice. If an animal can be genetically manipulated so that it contains a putative target that has been modified in such a way as to make it insensitive to an anaesthetic, but without affecting its behaviour in any other way, and this animal is found to be insensitive to that anaesthetic, then this constitutes direct evidence for the relevance of that target. This so-called 'knock-in' approach differs from the more conventional 'knock-out' approach, where animals are genetically manipulated such that individual proteins or protein subunits are not expressed. The obvious problem here is that knocking out a single gene can often cause numerous changes in the animal in addition to the one intended, which in this case is removing a putative anaesthetic target.

Finally, investigating the effects of 'specific' receptor inhibitors or activators on anaesthetic potency can be helpful, particularly for identifying targets that are *not* relevant. For example, if a putative target can be markedly inhibited without affecting anaesthetic potency, then it is unlikely to be relevant. The problem comes when the anaesthetic potency *is* affected. Here it is difficult to distinguish between a change in potency that occurs because a relevant target has been affected and a change in anaesthetic potency that occurs due to an effect on a parallel pathway which has no relevance to the action of the anaesthetic being tested.

A road map to understanding anaesthetic mechanisms – how propofol and etomidate act

Work on two intravenous general anaesthetics, propofol and etomidate, illustrate how these various criteria have been used to identify unambiguously their primary target, the $GABA_A$ receptor (see also Bowery & Smart, this issue). This receptor channel is found throughout the central nervous system, and is the most abundant, fast inhibitory, ligand-gated, ion channel in the mammalian brain. It is a member of the so-called 'cys-loop' superfamily of receptors because of a characteristic motif in the primary sequence. The receptor is formed from a pentamer of subunits (so far, six α, three β, one δ, one ε, one π and three ρ subunits have been cloned), with the vast majority ($>85\%$) being composed of $\alpha_1\beta_2\gamma_2$ (the most abundant), $\alpha_2\beta_3\gamma_2$ or $\alpha_3\beta_{1-3}\gamma_2$ combinations. The most predominant location for $GABA_A$ receptors is in the postsynaptic membrane, but extrajunctional $GABA_A$ receptors are also known to exist in many brain areas.

Pioneering work by several investigators, most notably Roger Nicoll, Jeff Barker, Bob Macdonald and Richard Olsen, had established by 1980 that the $GABA_A$ receptor was a likely target for anaesthetic barbiturates (Macdonald & Barker, 1978; Nicoll, 1978; Leeb-Lundberg *et al*., 1980). These studies showed that barbiturates could enhance GABAergic inhibitory responses, and that this occurred at the molecular level by allosterically modulating the $GABA_A$ receptor complex. Given the widespread distribution of the $GABA_A$ receptor and its likely role in many brain functions, its plausibility as an anaesthetic target was self-evident. During subsequent years, the case for the $GABA_A$ receptor being a key target for a variety of general anaesthetics was advanced, particularly by Adron Harris, Neil Harrison and Jerry Lambert. Prior to the widespread use of heterologous expression systems to study receptor pharmacology, there were generally two different approaches. One was to use electrophysiological techniques to study native tissue, where the molecular basis for some of the responses was often ill-defined, while the other was to measure the effects of anaesthetics on the binding of receptor-specific ligands, where only surrogate measures of function could be assessed. Electrophysiological approaches have proven, in my view, to have been the most influential, and definitive evidence that the $GABA_A$ receptor might be particularly sensitive to etomidate and propofol came from two such studies. Etomidate was shown (Proctor *et al*., 1986) to markedly

prolong the decay of GABAergic IPSPs in hippocampal slices, while having a relatively small effect on their amplitudes. The IPSC was prolonged by about four-fold by 10 μM etomidate; a similar effect was found with 100 μM pentobarbital. With propofol, low micromolar concentrations were shown (Hales & Lambert, 1991) to enhance greatly GABA-activated chloride currents in both chromaffin cells and spinal neurones. Subsequent estimates of anaesthetic concentrations of both propofol and etomidate of a few micromolar or less confirm that these effects on the $GABA_A$ receptor certainly satisfy the criterion of adequate sensitivity.

Propofol is a simple hindered phenol and does not contain a chiral carbon; so stereoselectivity cannot be used in this case as a way of assessing the relevance of a putative target. On the other hand, etomidate is a complex carboxylated imidazole with a single chiral carbon and therefore exists as two mirror-image enantiomers, an R(+) enantiomer which is used clinically and the less potent S(−) enantiomer. The ratio of potencies of the two enantiomers as general anaesthetics was mimicked by their potencies as GABA modulators, strongly supporting the case that the $GABA_A$ receptor was the key target (Tomlin *et al*., 1998). Also, both enantiomers were equally effective in disrupting lipid bilayers, adding a further nail in the coffin of lipid theories.

As $GABA_A$ receptors can be composed of many different combinations of subunits (although as mentioned above, a relatively limited number predominate *in vivo*), the next question was whether etomidate and propofol were more effective at some $GABA_A$ receptors than others. The development of heterologous expression systems such as *Xenopus* oocytes and HEK 293 cells allowed this question to be addressed, and there has been a bewildering amount of data on the effects of most general anaesthetics on many of the possible $GABA_A$ receptor subunit combinations that are likely to be physiologically relevant, as well as many that are not. It is impossible here to catalogue this body of work, but some relatively simple findings have emerged regarding the actions of etomidate and propofol. For both drugs it would appear that the β subunit plays a critical role. This is particularly clear in the case of etomidate, which has a roughly 10-fold greater effect on $GABA_A$ receptors that contain β_2 or β_3 subunits rather than β_1 subunits. This important discovery by Jerry Lambert and his colleagues (Hill-Venning *et al*., 1997) was followed within a year by two landmark papers published in the same month. One paper (Belelli *et al*., 1997) showed how the potentiating actions of etomidate could be greatly reduced when a single amino acid in the β_3 subunit (Asn289) was mutated to its β_1 equivalent (Ser). Impressively, when this β_1 Ser was replaced by Asn, the converse occurred and anaesthetic sensitivity was enhanced. The second paper (Mihic *et al*., 1997) used a sophisticated combination of chimeric receptors and site-directed mutagenesis to identify amino acids that were critical to the potentiating actions of alcohols and volatile anaesthetics. Subsequent work refined the importance of the various identified amino acids and it has become clear that different amino acids affect different anaesthetics in different ways. For the two intravenous agents I am considering here, it appears that, while the sensitivity of $GABA_A$ receptors to etomidate greatly depends upon the type of β subunit, propofol shows curiously little subunit dependence but, nonetheless, the same mutations in β_2 or β_3 subunits affect both anaesthetics (Siegwart *et al*., 2002).

The importance of this last point is that this has led to the most exciting development in the field for some years, the production of 'knock-in' mice (Jurd *et al*., 2003; Reynolds *et al*., 2003) that contain mutations in either β_2 or β_3 subunits that were expected, on the basis of the *in vitro* studies mentioned above (Figure 5a), to render the animals insensitive to etomidate, and/or propofol. The β_3(N265M) mice were dramatically less sensitive to both etomidate and propofol (Figure 5b) when the anaesthetic end point was withdrawal from a painful stimulus, while they retained their normal sensitivity to alphaxalone. These data reflected the *in vitro* data in a remarkable way and show that a single target is responsible for this anaesthetic end point (at least for etomidate and propofol). The mice were also less sensitive to etomidate and propofol when the duration of LORR was measured, but here by only a factor of about three. Interestingly, very similar reductions in durations of LORR were observed with the β_2(N265S) knock-in mice (using intravenous injections) as well as some reduction in responses to a painful stimulus. In addition, subanaesthetic concentrations of etomidate were less effective in reducing locomotor activity in the β_2(N265S) mice, but this was not investigated with the β_3(N265M) mice.

A significant problem with most of these *in vivo* experiments is that *times* for loss of a reflex were recorded, rather than the EC_{50} concentrations at which the reflex is just lost. This is an important distinction because pharmacokinetic factors become critically important, and sleep times will only indirectly reflect differences in target sensitivities. Nonetheless, this caveat aside, these papers represent a major advance from which it can be concluded that β_3-containing $GABA_A$ receptors are by far the most important target for etomidate and propofol for the loss of response to a painful stimulus (LHWR) and that the LORR caused by etomidate is mediated by both β_2- and β_3-containing $GABA_A$ receptors. Whether other targets are involved, such as β_1-containing $GABA_A$ receptors, cannot be assessed until a double knock-in is studied.

Our understanding of how propofol and etomidate act is more advanced than for any other anaesthetic, with the possible exception of dexmedetomidine, which is a highly selective α_2 adrenergic agonist with anaesthetic properties (Correa-Sales *et al*., 1992), but several key questions remain. At the molecular level, where are the binding sites on the $GABA_A$ receptor? Some indications have come from the labelling of substituted cysteines (Bali & Akabas, 2004), but perhaps this question will ultimately be answered using photoactivatable analogues (Husain *et al*., 2003). At the cellular level, are the relevant $GABA_A$ receptors synaptic or extrajunctional (Bai *et al*., 2001)? Finally, and perhaps most challenging of all, which neuronal structures are most important for the anaesthetic end points of interest? For example, are endogenous sleep pathways recruited (Nelson *et al*., 2002), and what is the balance of importance between spinal and supraspinal sites (Sonner *et al*., 2003)?

Inhalational general anaesthetics

In this short review, it is obviously impossible to cover all of the work carried out on trying to identify the most likely molecular targets for inhalational general anaesthetics, and I will therefore concentrate only on those that I think are most

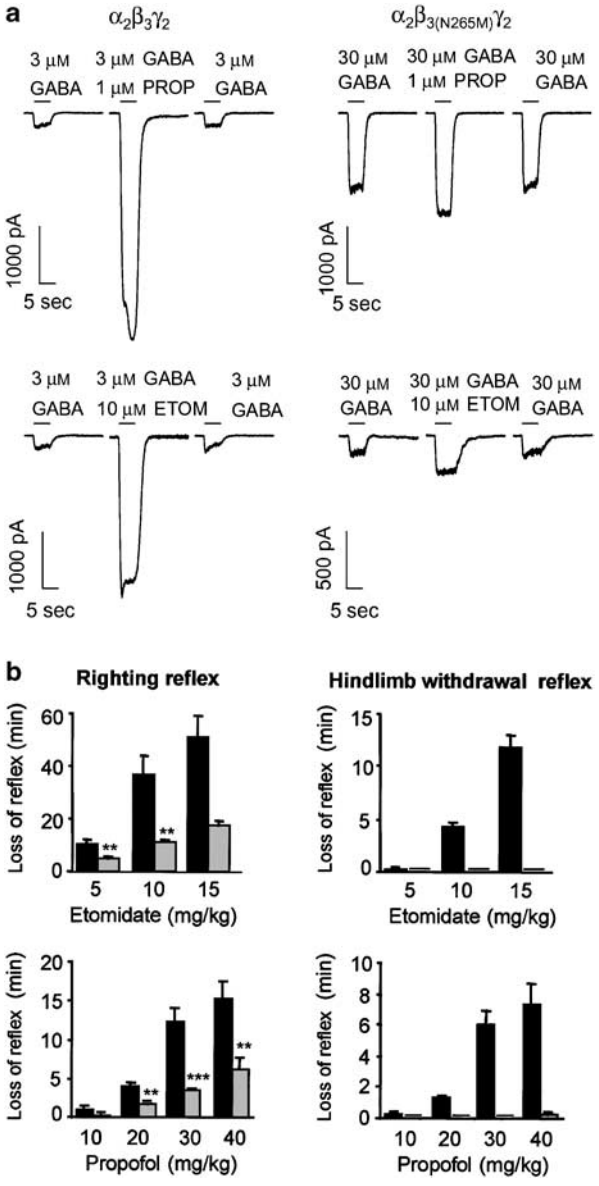

Figure 5 (a) *In vitro* data showing the potentiating actions of the intravenous anaesthetics, propofol (PROP) and etomidate (ETOM) on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors (Siegwart *et al*., 2002), and the effects of a β-subunit mutation, β_3(N265M). This mutation substantially, although not completely, reduces the potentiations by etomidate and propofol (but has little, if any, effect on the actions of alphaxalone – not shown). Reproduced with permission from Blackwell Publishing. (b) Behavioural responses to the same two anaesthetics in wild-type and β_3(N265M) knock-in mice (Jurd *et al*., 2003). Durations for LORR and LHWR were determined. ETOM and PROP had essentially no effect on LHWR in the β_3(N265M) mice and a reduced effect on LORR (corresponding to a rightward shift in the dose–response curve of about three-fold or less). Alphaxalone had the same effect for both end points in the wild-type and β_3(N265M) mice – not shown. Wild-type mice (black shading), β_3(N265M) mice (gray shading). $^{**}P<0.01$, $^{***}P<0.001$ compared to wild type. Reproduced with permission from FASEB.

important. For these drugs, probably more than any other, attention to pharmacologically relevant concentrations is critical because their relatively low selectivities mean that many targets are affected at concentrations only slightly in excess of those needed for anaesthesia. I believe that there are currently three prime candidates.

$GABA_A$ receptors

As potentiation of GABA acting on $GABA_A$ receptors containing β_2 and β_3 subunits is sufficient at least for certain anaesthetic end points (see above), and because many inhalational anaesthetics, such as halothane, isoflurane and sevoflurane, potentiate (Figure 6a) exactly the same receptors (see, e.g. Nishikawa *et al*., 2002), it seems more than reasonable that this will contribute to the anaesthesia caused by these agents. This is not the case for xenon and nitrous oxide, however, which show little, if any, activity at $GABA_A$ receptors. There are two caveats, however. The first is that the potentiation caused by volatile anaesthetics is usually smaller (typically half) than that observed by etomidate and propofol (at equi-anaesthetic concentrations), so $GABA_A$ receptors containing β_2 and β_3 subunits cannot be the only target. The second, more subtle, caveat is that actions at other sites could antagonise these effects (for example, activation of potassium channels in GABAergic interneurones).

Perhaps not surprisingly in view of the low potencies of volatile anaesthetics, stereoselectivity has provided less guidance as to which targets are most likely. Nonetheless, stereoselectivity has been observed in animals with isoflurane, although this might depend upon the end point, and a similar degree of stereoselectivity is found in potentiating the $GABA_A$ receptor.

The demonstration that specific amino acids were necessary for volatile anaesthetics to potentiate the $GABA_A$ receptor was an important breakthrough (Mihic *et al*., 1997). The first two key amino acids were identified in the α subunit – Ser270 and Ala291, but subsequently other amino acids have been identified which, when mutated, alter the sensitivity of the receptor to volatile anaesthetics. A plausibility case has been built up, which argues that some of these amino acids line an anaesthetic binding pocket (Jenkins *et al*., 2001). However, direct structural evidence for this is lacking, although the clever use of site-directed mutagenesis (specifically the introduction of cysteines at putative anaesthetic binding sites) and thiol reagents, which irreversibly bind to these amino acids, provides some support (Mascia *et al*., 2000). A major difficulty with most experiments that are designed to identify anaesthetic binding sites on these ligand-gated ion channels using chimeras, mutagenesis or chemical modification is their allosteric nature. Firstly, it is extremely difficult to distinguish between effects on gating and binding even under the most favourable circumstances, but secondly, when anaesthetics *enhance* activity, any binding site is almost certainly going to be a transduction site as well because binding must lead to changes in channel gating. The fact that specific mutations lead to effects on some anaesthetics but not others broadly supports the idea that anaesthetic binding sites are being disrupted, but the complexity of these receptor channels means that such a conclusion can only be considered to be tentative.

Volatile anaesthetics show no absolute requirement for any particular $GABA_A$ subunit for their potentiating effects and generally show little subunit dependence. This has meant that the knock-in approach that has proven so successful with etomidate (see above) is unlikely to be so straightforward for these agents. Nonetheless, the β_3(N265M) mice discussed

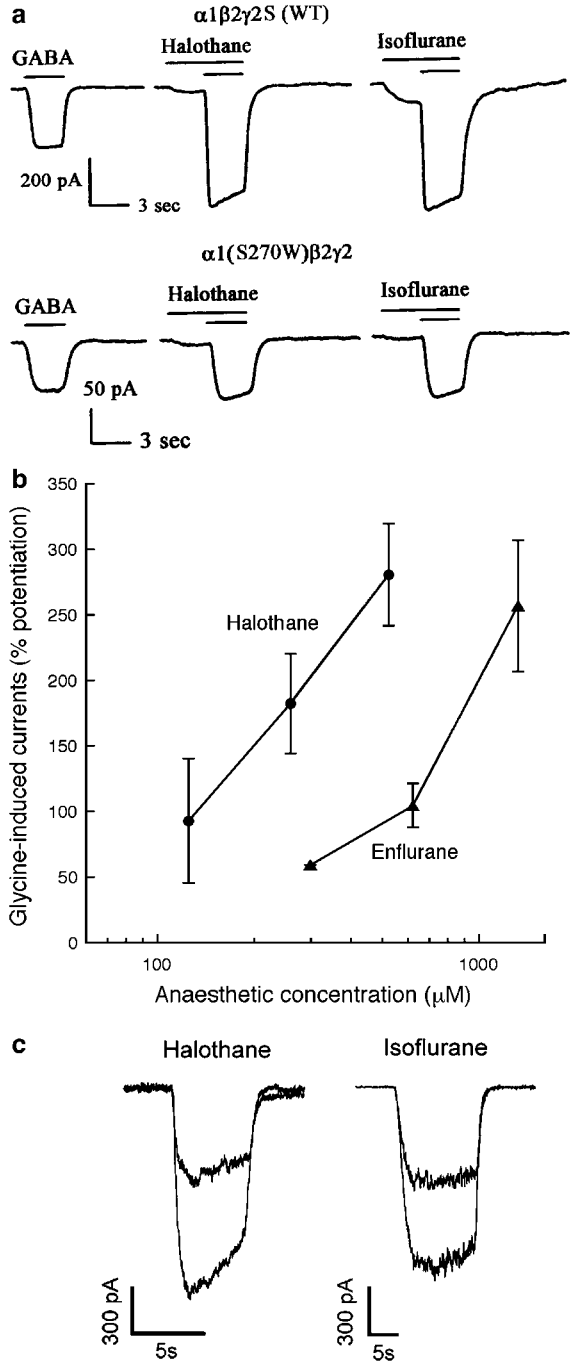

Figure 6 Two likely targets for inhalational general anaesthetics. (a) The $GABA_A$ receptor (Nishikawa *et al*., 2002). Reprinted with permission from Elsevier. These data show typical potentiations by halothane and isoflurane of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, and how these potentiations are virtually abolished by a single mutation in the α subunit (S270W). (b) The glycine receptor. These data show percentage potentiations by volatile agents acting on glycine receptors expressed in *Xenopus* oocytes (Mascia *et al*., 1996) and (c) in medullary neurons (Downie *et al*., 1996).

above show a significantly reduced sensitivity to halothane, enflurane and isoflurane in their responses to a painful stimulus, although there are no changes in their LORR (Lambert *et al*., 2005). These data reinforce the fact that anaesthetics are likely to recruit a different set of molecular targets for different anaesthetic end points, but also support the idea that $GABA_A$ receptors play some role in the actions of volatile agents.

Glycine receptors

Often colocalised with $GABA_A$ receptors, and of particular importance in lower brain centres and the spinal cord, glycine receptors are a highly plausible target (Figure 6b and c) for inhalational agents (Harrison *et al*., 1993; Downie *et al*., 1996; Mascia *et al*., 1996). Glycine receptors present a much more homogeneous population of targets than $GABA_A$ receptors because there are only four α subunits and a single β subunit, with substantial developmental changes resulting in the predominance of α_1 and β subunits in the adult. Glycine receptors can exist as $\alpha\beta$ heteromers or α homomers. Their plausibility as a target is particularly strong for the loss of response to a painful stimulus, which appears to be determined predominantly by actions in the spinal cord (Sonner *et al*., 2003). In both expression systems (Mascia *et al*., 1996) as well as neurons (Harrison *et al*., 1993; Downie *et al*., 1996), potentiation of glycine receptors is seen at low concentrations for all of the volatile agents, although there is no evidence of stereoselectivity (Downie *et al*., 1996).

So far genetic approaches have not been particularly productive, and definitive data proving the role of glycine receptors in general anaesthesia have yet to be published.

Two-pore-domain potassium channels

A new target for inhalational general anaesthetics has emerged relatively recently – the so-called two-pore-domain potassium channels. Anaesthetic-activated potassium channels were first characterised in the late 1980s in identified neurons of the pond snail *Lymnaea stagnalis* (Franks & Lieb, 1988) and their mammalian counterparts form a diverse family of channels that are characterised by having two pore-forming domains in their primary sequences, while having only moderate sequence homologies outside these regions. Among the 15 subunits so far identified, two subfamilies, TREK and TASK, have members that are activated by volatile and gaseous anaesthetics, although the activation is agent specific (Patel & Honore, 2001; see also Jenkinson, this issue). Most work has been carried out with halothane because it usually has the greatest effect. These anaesthetic-activated potassium channels are certainly plausible targets because they are thought to act as regulators of membrane excitability and they are, indeed, among the most richly modulated of all ion channels. They have complex distributions in the central nervous system and are found at both pre- and post-synaptic sites. Although the anaesthetic activations are never very large at clinically relevant concentrations, there is good evidence that they may be sufficient to cause substantial changes in neuronal excitability. For example, excellent work by Doug Bayliss and his colleagues has shown that halothane reduces firing activity by hyperpolarising neurons by activating a TASK-like current (Figure 7a and b) at sites that may well play a role in anaesthesia, such as the locus coeruleus and the hypoglossal nucleus (Sirois *et al*., 2000).

Perhaps the strongest case has been made for the role of TREK-1, based on data from a knock-out mouse from Michel Lazdunski's laboratory (Heurteaux *et al*., 2004). This work

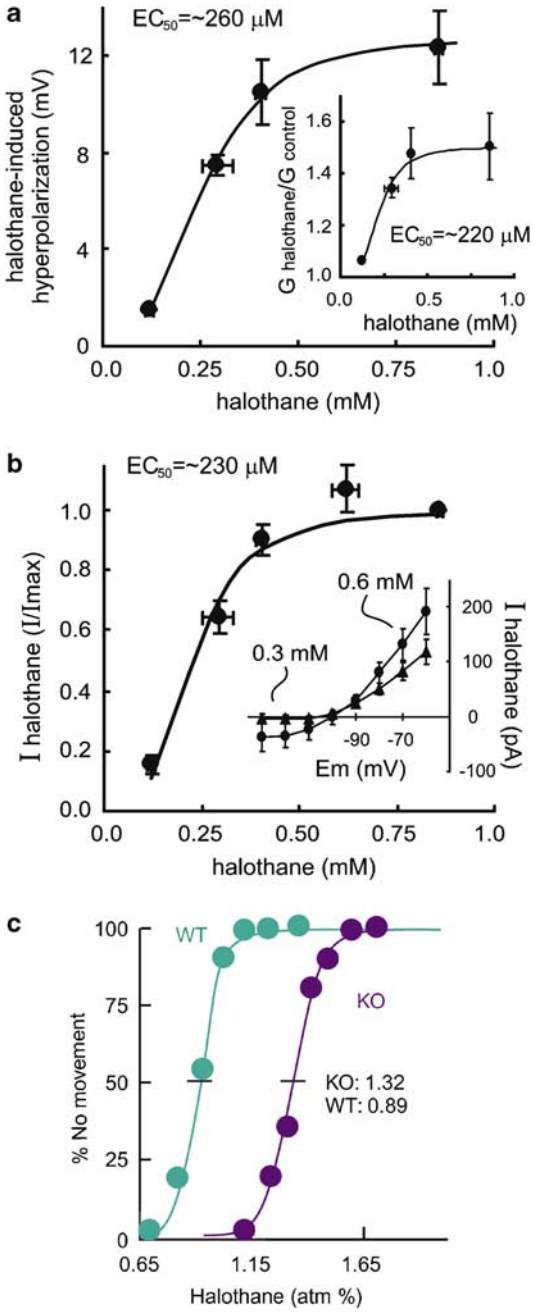

Figure 7 The most recently identified target for inhalational anaesthetics – two-pore domain potassium channels. (a) These data are from rat motoneurons showing the extent of halothane-induced hyperpolarisation and (b) halothane-activated currents due to the anaesthetic activation of a TASK-like potassium channel (Sirois *et al*., 2000). Copyright 2000 by the Society of Neuroscience. (c) *In vivo* data showing how TREK-1 knock-out mice are significantly less sensitive to halothane than wild-type controls (Heurteaux *et al*., 2004). Reprinted with permission.

was the culmination of a series of studies from Patel & Honore (2001) and their colleagues from the same laboratory which had shown that TREK-1 was sensitive to a wide variety of inhalational anaesthetics, and had identified the C-terminal domain as an important modulatory region for anaesthetic action (as well as most other modulatory influences). The knock-out animals were considerably less sensitive in their responses to a painful stimulus (Figure 7c), but only slightly less sensitive when LORR was measured. An important control showed that the animals were equally sensitive to pentobarbital (which does not activate TREK-1), but this was only looked at in the LORR assay. As with all knock-out data, one has to be cautious because, as mentioned above, many compensatory changes can occur. In this study, an impressive array of tests were performed to look for this, and the authors concluded that the phenotypic changes observed could be interpreted as being due simply to the absence of TREK-1 channels. Data on knock-in animals, if they can be devised, or on conditional knock-outs where TREK-1 expression is eliminated only in certain anatomical sites will be required before these exciting data on TREK-1 knock-out animals can be considered as the final word on the role of this channel in general anaesthesia.

Other possible targets for inhalational anaesthetics

The number of different targets that have been proposed over the years as targets for inhalational agents is very large (Franks & Lieb, 1994; Campagna *et al*., 2003; Sonner *et al*., 2003), although many can safely be discounted because effects are often only seen at unreasonably high concentrations. Some targets that I would class as interesting, but as yet unproven, include NMDA receptors (which seem a likely target for nitrous oxide and xenon), HCN channels (Chen *et al*., 2005) and some subtypes of the sodium channel (Wu *et al*., 2004). There is unfortunately no space here to consider these possibilities, but hopefully definitive data will emerge in the next few years which bear on their relevance.

Concluding remarks

In this review, I have described how the field of anaesthetic mechanisms has changed dramatically over the last 30 years. The days when anaesthetics were thought to act nonspecifically by simply dissolving in membranes are long gone. The full spectrum of sophisticated techniques available to the pharmacologist has been brought to bear on one of the most intriguing class of drugs – those that remove consciousness – and have shown a degree of specificity that was undreamt of only a few years ago. As far as which molecular targets are responsible, I have concentrated on those anaesthetics (such as propofol and etomidate) where we have the most complete picture, together with those about which we know least (the inhalational agents). I have barely mentioned those anaesthetics that sit in between (such as the barbiturates) about which we know a great deal, but not the whole story. I think in the next few years the issue about which targets are important and which are not will have been essentially resolved for most, if not all, anaesthetics. The next set of questions about where these targets are located at the cellular and anatomical levels and how these drugs affect their changes at the molecular level will keep those interested in this branch of pharmacology busy for many years to come.

I would like to dedicate this article to the late Bill Lieb, with whom I collaborated for over 30 years. I thank the MRC for support and Robert Dickinson for helpful comments on the manuscript.

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