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Actions of general anaesthetics on 5-HT_3 receptors in N1E-115 neuroblastoma cells

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1 N1E-115 mouse neuroblastoma cells were studied under voltage clamp in the whole-cell patch-clamp configuration. Peak currents induced by bath application of 5-hydroxytryptamine (5-HT) were inwardly rectifying, reversed at 0.4 ± 0.2 mV (mean ± s.e.mean), and were approximately half-inhibited (at 1 μM 5-HT) by 2 nM of the 5-HT_3 selective antagonist MDL-72222 (3-tropanyl-3,5-dichlorobenzoate).

2 Peak inward currents activated by a low concentration of 5-HT at a holding potential of −50 mV were potentiated by volatile general anaesthetics. At their human minimum alveolar concentrations (MACs), the degree of potentiation increased in the order isoflurane < halothane < enflurane < methoxyflurane. Potentiation by methoxyflurane was independent of membrane potential in the range −70 mV to +40 mV. The reversal potential was the same in the presence and absence of methoxyflurane.

3 Methoxyflurane shifted the 5-HT dose-response curve to lower 5-HT concentrations, without significantly changing the Hill coefficient or maximum response. The EC_{50} concentration for 5-HT decreased from 1.86 ± 0.02 μM to 1.07 ± 0.11 μM (means ± s.e.mean) due to the presence of 1 MAC (270 μM) methoxyflurane.

4 In contrast to the volatile anaesthetics, the barbiturate anaesthetic, thiopentone, inhibited the 5-HT_3 receptor. Hill analysis of thiopentone dose-response data gave an average $IC_{50} = 117 \pm 8$ μM thiopentone and Hill coefficient = 1.6 ± 0.2 (means ± s.e.mean). These parameters were not significantly different for data obtained at 5-HT concentrations above and below the control EC_{50} concentration for 5-HT, consistent with non-competitive inhibition.

5 The *n*-alcohols occupied an intermediate position between the volatile and barbiturate anaesthetics. The lower alcohols (butanol and hexanol) potentiated 5-HT responses at low alcohol concentrations but inhibited them at high concentrations. In contrast, the higher alcohols (octanol, decanol, dodecanol, tridecanol, tetradecanol and pentadecanol) produced no potentiation, but only inhibition, at all alcohol concentrations.

6 Inhibition of the 5-HT_3 receptor by the *n*-alcohols exhibited a cutoff in potency similar to those previously found for tadpoles, luciferase enzymes and a neuronal nicotinic acetylcholine receptor channel.

Keywords: General anaesthesia; 5-HT_3 receptor; 5-hydroxytryptamine; inhalational anaesthetics; barbiturates; alcohols; N1E-115 neuroblastoma cells; cutoff effect

Introduction

The 5-HT_3 receptor is a member of the genetically related superfamily of ligand-gated receptor-channels that also includes the $GABA_A$ receptor, the glycine receptor, and neuronal and muscle-type nicotinic acetylcholine (ACh) receptors (Unwin, 1993; Ortells & Lunt, 1995). Members of this superfamily, most notably the nicotinic ACh and $GABA_A$ receptors, are amongst the most sensitive of all known ion channels to general anaesthetics (Franks & Lieb, 1994; McKenzie *et al.*, 1995) and are currently the most plausible candidates for the primary targets underlying general anaesthesia (Franks & Lieb, 1994). Members of this superfamily have homologous subunits and appear to share a basic pentameric structure, with five membrane-spanning M2 regions (one from each subunit) forming the central aqueous channel or pore through which ions pass (Maricq *et al.*, 1991; Unwin, 1993; Boess *et al.*, 1995). Functional homomeric 5-HT_3 receptors with properties similar to native receptors can be formed from a single type of subunit (Maricq *et al.*, 1991; Hope *et al.*, 1993; Hussy *et al.*, 1994; Werner *et al.*, 1994; Gill *et al.*, 1995), suggesting a relatively simple structure for the native receptors. Furthermore, a recent molecular evolution analysis has concluded that the 5-HT_3 receptor is one of the most primitive members of this superfamily of ligand-gated receptor-channels (Ortells & Lunt, 1995). Clinically, the 5-HT_3 receptor may play some role in producing general anaesthesia, and it may also cause some of the unpleasant side-effects associated with general anaesthesia, such as the postoperative nausea and vomiting which can be alleviated with 5-HT_3 receptor antagonists such as ondansetron (Haigh *et al.*, 1993).

Previous electrophysiological studies of the effects of general anaesthetics on the 5-HT_3 receptor have mostly been carried out in the context of alcoholism and have therefore focused on the potentiating effects of ethanol and other lower alcohols (Lovinger, 1991; Lovinger & White, 1991; Lovinger & Zhou, 1993; 1994; Downie *et al.*, 1995). There have been no publications on the effects of the higher alcohols, and only a handful of electrophysiological studies on the effects of other general anaesthetics (Peters *et al.*, 1991; Machu & Harris, 1994; Downie *et al.*, 1995). In the work described here, we have looked at the effects on the 5-HT_3 receptor of four volatile general anaesthetics (enflurane, halothane, isoflurane, and methoxyflurane), eight *n*-alcohols (butanol, hexanol, octanol, decanol, dodecanol, tridecanol, tetradecanol and pentadecanol) and one barbiturate (thiopentone). We chose to study the 5-HT_3 receptor from the mouse neuroblastoma cell line N1E-115, which expresses two closely related types of 5-HT_3 re-

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ceptor subunits (Hope *et al.*, 1993). An advantage of using this neuronal cell line is that application of 5-HT to these cells activates only the 5-HT$_3$ subtype of 5-HT receptors (Neijt *et al.*, 1988; Lambert *et al.*, 1989; Hussy *et al.*, 1994). Furthermore, 'giga-ohm' seals can easily be formed to these cells, making it possible to study 5-HT responses in the whole-cell patch-clamp configuration under constant membrane potential, where 5-HT-activated currents directly reflect 5-HT$_3$ channel activity.

Methods

Cell culture

Cryopreserved N1E-115 mouse neuroblastoma cells (Amano *et al.*, 1972), passage number 18, were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire). The cells were resuscitated and then cultured (at 37°C in an atmosphere of 95% air/5% CO_2 at 95% relative humidity) in culture medium, which consisted of Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated foetal calf serum, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and L-glutamine (2 mM), all obtained from Gibco BRL Life Technologies Ltd. (Paisley, Scotland). Cells were subcultured (passaged) every week; cells used in these experiments had passage numbers between 21 and 50. For the patch-clamp experiments, cells were seeded onto glass coverslips (cut with a diamond knife into strips of $\sim$1.5 mm $\times$ 9 mm). Seeding was achieved by placing the coverslips in six-well dishes (Greiner Labortechnik Ltd., Cam, Gloucestershire) and covering them with 4 ml of a suspension of cells (4×10^4 cells ml^{-1}) in plating medium, which was culture medium with the concentration of heat-inactivated foetal calf serum reduced from 10% to 2% (vol/vol) in order to reduce the growth rate and hence minimize cell aggregation. No differentiating agents (e.g. DMSO or dibutyl-cyclic AMP) were added to the plating medium. Cells on the coverslips were incubated for 1 to 5 days before use.

Solutions for electrophysiology

The composition of normal extracellular saline was (mM): NaCl 120, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.8, HEPES 20, glucose 20; titrated to pH 7.4 with NaOH. The composition of the internal recording solution inside the patch pipettes was (mM): CsCl 145, NaCl 2.5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, EGTA 10; titrated to pH 7.2 with CsOH. 5-HT solutions were made up on the day of the experiment, using the 5-HT/creatinine sulphate complex. All of these chemicals were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset).

Anaesthetics were dissolved in normal extracellular saline, with or without 5-HT. The volatile anaesthetics were made up as fractions of saturated solutions at room temperature. The concentrations of the saturated solutions were assumed to be: methoxyflurane, 9.1 mM (Seto *et al.*, 1992); enflurane, 11.9 mM (Seto *et al.*, 1992); isoflurane, 15.3 mM (Franks & Lieb, 1991); halothane, 17.5 mM (Raventós, 1956). Thiopentone, *n*-butanol, *n*-hexanol and *n*-octanol were added directly or diluted from stock solutions in normal extracellular saline. The higher *n*-alcohols (decanol and above) were prepared using ethanolic stock solutions. The final ethanol concentration was less than 20 mM (except for the highest dodecanol concentration, where it was 26 mM), and an identical concentration of ethanol was present in the equivalent control solutions. MDL-72222 was prepared using a stock solution in DMSO (Sigma), with a final DMSO concentration $\leqslant$ 175 μM.

Sources of anaesthetics and antagonists

The sources of the anaesthetics were as follows: isoflurane, enflurane, methoxyflurane (Abbott Laboratories Ltd., Queenborough, Kent); halothane (ICI Ltd., Macclesfield, Cheshire); thiopentone (supplied as 92% thiopentone/8% Na_2CO_3 by May and Baker, Dagenham, Essex); ethanol, *n*-butanol, *n*-hexanol, *n*-octanol, *n*-decanol, *n*-dodecanol (BDH Chemicals Ltd., Poole, Dorset); *n*-tridecanol (Sigma); *n*-tetradecanol, *n*-pentadecanol (Aldrich Chemical Co. Ltd., Gillingham, Dorset). The selective 5-HT$_3$ antagonist, MDL-72222 (3-tropanyl-3,5-dichlorobenzoate) was obtained from Research Biochemicals International (RBI, Natick, Massachusetts, U.S.A.).

Electrophysiology

Electrophysiological measurements were made by the whole-cell patch-clamp technique (Hamill *et al.*, 1981), with methods similar to those described previously (Hall *et al.*, 1994). Micropipettes were fabricated from thin-walled filamented borosilicate glass capillary tubes (GC150TF, Clark Electromedical Instruments, Reading, Berkshire) using a two-stage pull (Narishige PB-7 micropipette puller, Tokyo, Japan), lightly fire-polished and filled with internal recording solution (see above). Electrode resistances were between 1–5 MΩ. 'Giga-ohm' seals were made to N1E-115 cells attached to a glass coverslip and bathed in normal extracellular saline. Cells were chosen that were isolated from other cells, that had soma diameters $<$ 40 μm, and that possessed a few short processes. After achieving the whole-cell configuration, cells were voltage-clamped at $-$50 mV (the standard holding potential) and their cytoplasm left to equilibrate with the electrode solution for about 10 min before recording. Recordings were made with an Axopatch 200 amplifier (Axon Instruments, Foster City, California, U.S.A.). Series resistance was compensated by 60–90%. The current record was filtered (5 Hz, $-$3 dB) by an 8-pole Bessel filter. The current and voltage records were digitized and stored on a computer prior to analysis.

Cells in the bath (1.5 mm deep, 4 mm wide, 30 mm long) were placed near the inlet and continuously perfused ($\sim$1 ml min^{-1}) with either control or test solutions, delivered by gravity feed from one of eleven glass reservoirs. Reservoirs containing volatile anaesthetics were sealed with a rigid plastic float, and all tubing and valves were made of PTFE. With these precautions, losses of volatile agents from the perfusion system were found to be negligible when measured by gas chromatography (Hall *et al.*, 1994). The degree of anaesthetic potentiation or inhibition was determined as follows. Two or more control 5-HT responses were followed by two or more responses to the simultaneous application of both 5-HT and anaesthetic, and these in turn were followed by two or more additional control 5-HT responses. The mean of the peak anaesthetic responses was then compared to the mean of all the peak control responses (both before and after application of anaesthetic). The only exception to this procedure occurred with the higher *n*-alcohols, where, although the degree of inhibition was reproducible, the post-anaesthetic controls often showed only limited reversibility; for these cases, the post-anaesthetic controls were excluded in the calculation of the degree of inhibition. Responses to anaesthetics in the absence of 5-HT were found to be negligible. On a longer time scale, there was a slow rundown in control 5-HT response, such that the peak response after $\sim$80 min was about half the initial response. This was a problem only for the 5-HT dose-response experiments, and it was corrected for by recording multiple control responses at the same 5-HT concentration throughout the course of the experiments. All experiments were performed at room temperature (21–23°C).

Values are given as means $\pm$ s.e.mean.

Results

The control 5-HT-activated current

It is well-established that application of 5-HT to N1E-115 neuroblastoma cells selectively activates 5-HT$_3$ receptor

channels (Neijt *et al.*, 1988; Peters *et al.*, 1988; Lambert *et al.*, 1989; Hussy *et al.*, 1994). We studied 5-HT responses under whole-cell voltage clamp and confirmed that the 5-HT-activated currents were reversibly inhibited by low concentrations of the potent 5-HT$_3$ receptor antagonist, MDL-72222, with an $IC_{50} \approx 2$ nM ($n = 4$ cells; see also upper left inset to Figure 1). Furthermore, in normal extracellular saline the currents had a reversal potential (mean ± s.e.mean, $n = 5$ cells) of 0.4 ± 0.2 mV (see also Figure 3), which is not significantly different from zero, consistent with the opening of a non-selective cation channel. The 5-HT-activated currents desensitized in the continued presence of agonist, even at low concentrations (≥500 nM). The apparent rates of both activation and desensitization increased with increasing agonist concentrations (see insets to Figure 1) but were not studied further due to the relatively low time resolution of our perfusion system. The peak responses were dose-dependent and could be well-fitted by a Hill equation of the form $y = c^{nH}/(c^{nH} + EC^{nH}_{50})$, where y is the current expressed as a fraction of the maximal current, c is the 5-HT concentration, n_H is the Hill coefficient, and EC_{50} is the 5-HT concentration giving a half-maximal response (see Figure 1). The least squares fit gave (means ± s.e.means) an EC_{50} of 1.86 ± 0.02 μM 5-HT and a Hill coefficient of 2.03 ± 0.04, consistent with at least two agonist molecules being required for activation of the 5-HT$_3$ receptor channel.

Potentiation of 5-HT responses by volatile general anaesthetics

Volatile anaesthetics potentiated the response to low concentrations of 5-HT, as shown in Figure 2 for methoxyflurane, enflurane, halothane and isoflurane. The degree of potentiation varied considerably from agent to agent. At the human minimum alveolar concentration (MAC) for each agent, the degree of potentiation increased in the order isoflurane < halothane < enflurane < methoxyflurane. Since methoxyflurane gave the largest potentiation, it was chosen for more detailed study.

We first studied the voltage-dependence of control and methoxyflurane-potentiated currents. Representative current traces from a single cell are shown in Figure 3a, and mean peak responses from a large number of cells are plotted in Figure 3b. Notice that the currents were inwardly rectifying. It is clear from Figure 3 that the degree of potentiation was essentially independent of transmembrane potential in the range from −70 mV to +40 mV. Furthermore, the reversal potential (mean ± s.e.mean, $n = 5$ cells) was not significantly different for control and anaesthetic-potentiated currents (0.4 ± 0.2 mV and 0.6 ± 1.1 mV, respectively).

Whereas clinically relevant concentrations of methoxyflurane could greatly potentiate the response to low concentrations of 5-HT, this was not true at very high 5-HT concentrations. This is shown in Figure 4, where it can be seen that a concentration (270 μM = human MAC) of methoxyflurane that more than doubled the peak response to 1 μM 5-HT had little effect on the current activated by 50 μM 5-HT. We followed up this observation by determining the 5-HT dose-response relationship at different concentrations of methoxyflurane. The results are plotted in Figure 5, which shows that the major effect of methoxyflurane was to shift the 5-HT dose-response curve to the left in a dose-dependent manner. Unweighted least squares fits of the data to a Hill equation gave (means ± s.e.mean) EC_{50} concentrations of 1.86 ± 0.02, 1.36 ± 0.10 and 1.07 ± 0.11 μM 5-HT and Hill coefficients n_H of 2.03 ± 0.04, 1.64 ± 0.23 and 1.81 ± 0.30 for data at 0, 90 and 270 μM methoxyflurane, respectively. Thus methoxyflurane at its human MAC increased the apparent affinity of the 5-HT$_3$ receptor channel for 5-HT without significantly changing the Hill coefficient.

There was a significant increase in the rate of desensitization of the 5-HT response when it was potentiated by methoxyflurane (data not shown). Although we did not investigate this effect in any detail, it appeared that the degree of desensitization could be accounted for in terms of the extent to which the current had been activated. In other words, control currents of an equivalent size showed approximately the same rate of de-

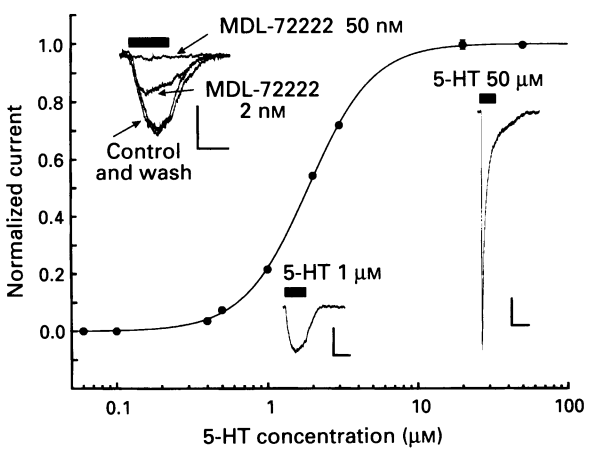

Figure 1 The 5-HT dose-response curve. The data points (●) give the mean peak currents as a function of 5-HT concentration. The errors are s.e.mean and were not shown where smaller than the size of the symbols. The line is an unweighted least squares fit of 92 individual determinations (from 15 cells) to a Hill equation, with $EC_{50} = 1.86 \pm 0.02$ μM and the Hill coefficient $n_H = 2.03 \pm 0.04$. The central and right insets show current traces from the same cell (voltage-clamped at −50 mV) in response to 1 μM and 50 μM 5-HT, respectively. The current traces in the upper left inset, from another cell, show inhibition of the response to 5-HT (1 μM) by MDL-72222, a selective antagonist for the 5-HT$_3$ receptor channel. Agonist and antagonist were applied together. Notice that the peak current was half-inhibited in a reversible manner by a low (2 nM) concentration of MDL-72222. The 5-HT response was almost completely abolished (though not as reversibly: not shown) by 50 nM MDL-72222. The solid bars in the insets show the periods of drug administration, while the calibration lines refer to 100 pA and 50 s.

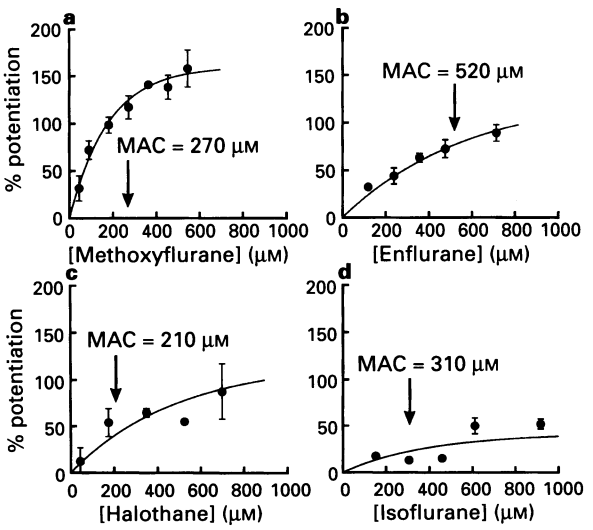

Figure 2 Anaesthetic dose-response relationships for potentiation of 5-HT peak responses by four volatile general anaesthetics: (a) methoxyflurane, (b) enflurane, (c) halothane, (d) isoflurane. For each agent, the arrow indicates the human minimum alveolar concentration (MAC), as tabulated in Table 1 of Franks & Lieb (1993). The 5-HT concentration was 1 μM. The lines were drawn by eye and have no theoretical significance. The data points (●) are plotted as means ± s.e.mean for 4–14 observations on 3 - 10 cells. Where not shown, the errors were smaller than the size of the symbols.

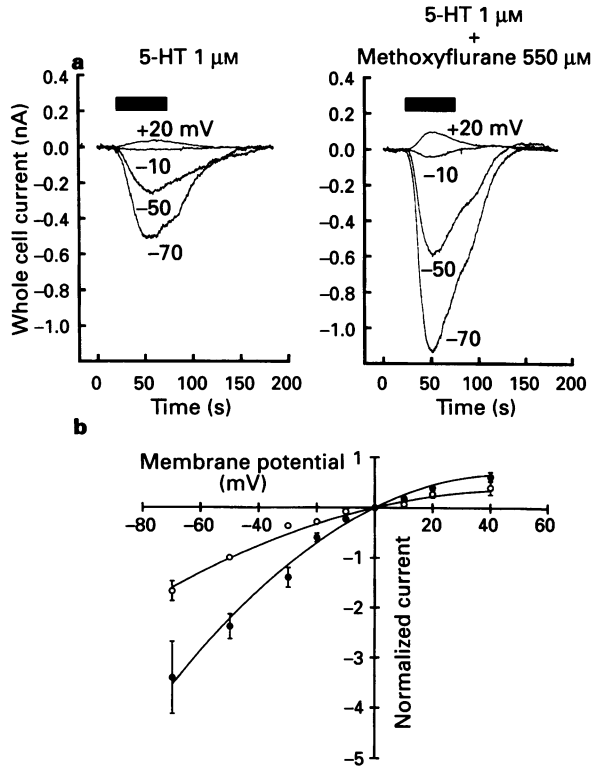

Figure 3 Effect of membrane potential on control and methoxyflurane-potentiated currents activated by a low concentration (1 μM) of 5-HT. When present, the methoxyflurane concentration was 550 μM. (a) Current traces for (left) control and (right) methoxyflurane-potentiated responses from a single cell. Holding potentials (from top to bottom) were +20 mV, −10 mV, −50 mV and −70 mV. (b) Peak-current-voltage relationships for (○) control and (●) methoxyflurane-potentiated responses. Data from 10 different cells were normalised by setting control currents at −50 mV to an arbitrary value of −1. Each error is the s.e.mean of at least 4 determinations from at least 3 cells and where not shown was smaller than the size of the symbol. The lines were drawn by eye. Notice that there is no significant voltage-dependence of the anaesthetic potentiation. Reversal potentials, calculated from a linear fit to the data in the range −10 to +10 mV, were 0.4±0.2 mV for the control currents and 0.6±1.1 mV for the currents in the presence of anaesthetic (mean±s.e.mean for $n=5$ cells).

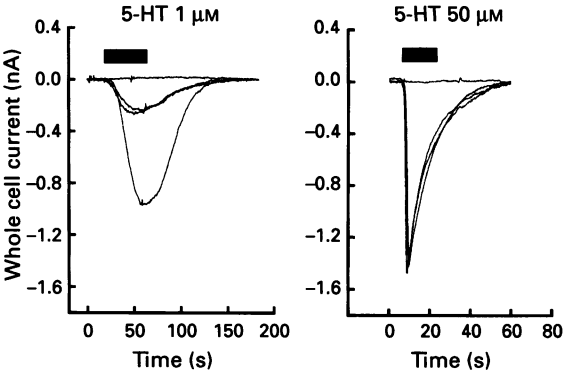

Figure 4 Large potentiation by methoxyflurane (1 MAC) of the response to a low (1 μM) but not to a high (50 μM) 5-HT concentration. Current traces for each cell were obtained in the following order: 5-HT alone, methoxyflurane alone, methoxyflurane + 5-HT applied together, 5-HT alone. When present, methoxyflurane was applied at a concentration of 270 μM. The 'baseline' currents show the lack of response to methoxyflurane on its own. Notice the large potentiation by methoxyflurane at 1 μM 5-HT, and the absence of any appreciable potentiation at 50 μM 5-HT. The cells were clamped at −50 mV (the standard holding potential).

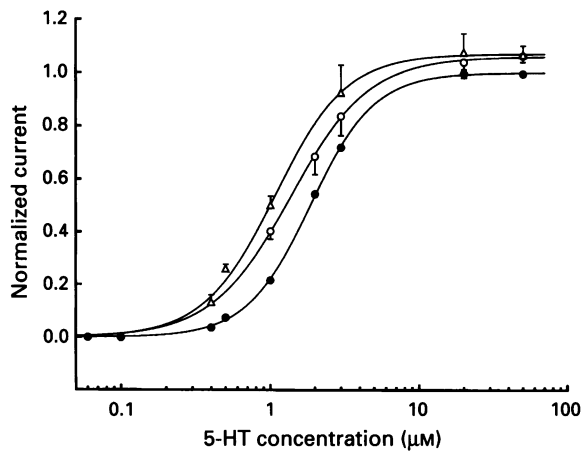

Figure 5 5-HT dose-response curves at different methoxyflurane concentrations: (●), 0 μM; (○), 90 μM; (△), 270 μM. The control dose-response curve is that shown in Figure 1. Each anaesthetic data point was obtained by multiplying the control response at the relevant 5-HT concentration by the observed degree of potentiation. The displayed points are the means±s.e.means of at least 4 observations on at least 3 cells; where not shown, the errors were smaller than the size of the symbols. The lines are unweighted least squares fits of the data to a Hill equation with EC_{50} concentrations of 1.86±0.02, 1.36±0.10 and 1.07±0.11 μM 5-HT, Hill coefficients of 2.03±0.04, 1.64±0.23 and 1.81±0.30, and relative maximum responses of 1.000±0.005, 1.06±0.03, and 1.07±0.04, for data at 0, 90 and 270 μM methoxyflurane, respectively.

sensitization. This is consistent with the lack of an effect of methoxyflurane on the desensitization of a maximal response to 5-HT (see Figure 4).

Inhibition of 5-HT responses by thiopentone

In contrast to the volatile anaesthetics, which potentiated responses at low concentrations of 5-HT, thiopentone (a widely used intravenous anaesthetic) inhibited 5-HT responses. The thiopentone dose-response curve at 1 μM 5-HT is shown in Figure 6. The data were fitted to a Hill equation of the form $y = 100\ IC^{n_H}{}_{50}/(c^{n_H} + IC^{n_H}{}_{50})$, where y is the current expressed as a percentage of the control current in the absence of anaesthetic, c is the thiopentone concentration, n_H is the Hill coefficient, and IC_{50} is the thiopentone concentration giving 50% inhibition. The least squares fit gave (means±s.e.mean) an IC_{50} of 115±10 μM thiopentone and a Hill coefficient n_H of 1.67±0.27. At 3 μM 5-HT, the corresponding values were IC_{50} = 119±13 μM thiopentone and n_H = 1.48±0.23 (data not shown), which are not significantly different from the values at 1 μM 5-HT. These IC_{50} concentrations can be compared to the much lower concentrations ($EC_{50} \approx 25$ μM thiopentone for lack of response to a painful stimulus) sufficient to produce general anaesthesia in mammals (Franks & Lieb, 1994).

Inhibition of 5-HT responses by the n*-alcohols*

The *n*-alcohols were found to occupy an intermediate position between the volatile and barbiturate anaesthetics. The lower *n*-alcohols (butanol and hexanol) potentiated 5-HT responses at low alcohol concentrations but inhibited them at higher concentrations, whereas the higher *n*-alcohols (octanol, decanol, dodecanol, tridecanol and tetradecanol) inhibited responses at all alcohol concentrations studied (see Figure 7). Previous alcohol studies (Lovinger, 1991; Lovinger & White, 1991; Lovinger & Zhou, 1994; Machu & Harris, 1994; Downie *et al.*, 1995) on the 5-HT$_3$ receptor have looked at the potentiating effects of the lower alcohols (including ethanol) but have not reported inhibitory effects for either lower or higher alcohols.

We therefore focused our attention upon these novel inhibitory effects. In order to reduce potentiating effects (for the lower alcohols in particular), all alcohol experiments were performed at 3 μM 5-HT.

The *n*-alcohol dose-response data are shown in Figure 7, where the current in the presence of alcohol, expressed as a percentage of control current, is plotted against the logarithm of alcohol concentration. (We found that inhibition by the highest concentrations of the higher *n*-alcohols was often not fully reversible, even after up to 30 min of washout. This effect was not studied further.) Notice the potentiation at low levels of butanol and hexanol and the inhibitions at high levels, together with the wholly inhibitory effects of the higher alcohols from octanol upwards. When the potentiating effects of butanol and hexanol were ignored, Hill analysis of the resulting data (see Table 1) showed that the Hill coefficients did not differ significantly from butanol to tridecanol (that is, the inhibitory curves were essentially parallel). Nearly saturated solutions of the two highest *n*-alcohols studied, tetradecanol and pentadecanol, produced some inhibition but could not produce 50% inhibition. [We found that a 98% saturated solution of *n*-pentadecanol produced only 15±2% inhibition (mean±s.e.mean for 4 cells).] An IC_{50} for tetradecanol was obtained by extrapolation (see dashed curve in Figure 7) of data with inhibitions up to 35% (for concentrations up to 92% of a saturated solution) and is listed together with the other IC_{50} concentrations in Table 1.

Discussion and conclusions

The volatile general anaesthetics

All four volatile anaesthetics potentiated responses to a low concentration (1 μM) of 5-HT, and this occurred at clinically relevant anaesthetic concentrations (see Figure 2). At the human MAC for each agent, potentiations greater than 50% were observed for methoxyflurane, enflurane and halothane, but a much smaller potentiation (13±4%, mean±s.e.mean of 17 observations on 10 cells) was observed for isoflurane. We

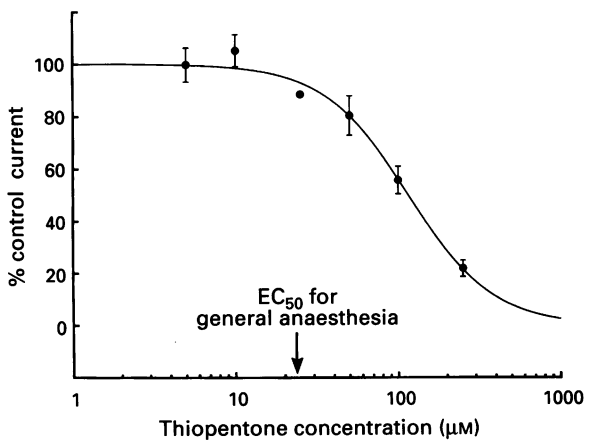

Figure 6 Thiopentone dose-response relationship for inhibition of peak responses to 1 μM 5-HT. The data points (●) and error bars give the ratios (expressed as percentages) of inhibited to control currents (mean±s.e.mean for 3-5 cells) as a function of thiopentone concentration. The point at 25 μM was a single determination. The line is the unweighted least squares fit of 24 individual determinations to a Hill equation (see text), with an IC_{50} of 115±10 μM and a Hill coefficient of 1.7±0.3. The arrow indicates the EC_{50} for mammalian general anaesthesia (Franks & Lieb, 1994). Notice that, even though a low agonist concentration was used to maximize any possible potentiation by thiopentone, no significant potentiation was observed.

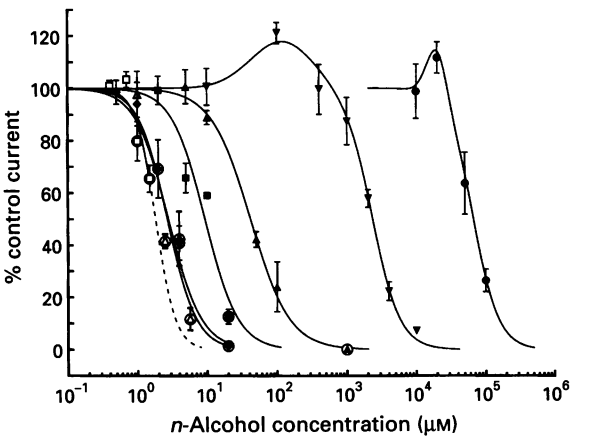

Figure 7 *n*-Alcohol dose-response relationships for modulation of responses to 3 μM 5-HT. Each data point gives the ratio, expressed as a percentage (mean±s.e.mean for at least 3 cells), of peak current in the presence and absence of each *n*-alcohol as a function of alcohol concentration (μM). The symbols are: (●) butanol; (▼) hexanol; (▲) octanol; (■) decanol; (◆) dodecanol; (△) tridecanol; (□) tetradecanol. Circled symbols denote alcohol inhibitions that were not fully reversible. Where error bars are not present, they were smaller than the size of the symbols. Each line is the unweighted least squares fit of the data to an inhibitory Hill equation (see text), with the exception of the lines for butanol and hexanol which were drawn by eye. The dashed portion of the line for tetradecanol is an extrapolation from data obtained below 1.4 μM (92% of its aqueous solubility).

Table 1 IC_{50} concentrations and Hill coefficients for inhibition of the 5-HT$_3$ receptor and EC_{50} concentrations for general anaesthesia

	Inhibition of 5-HT$_3$ receptor			*General anaesthesia*	
Anaesthetic	*IC_{50} (μM)*	*Hill coefficient*	*d.f.*	*EC_{50}*	*Animal*
Thiopentone	119±13	1.48±0.23	26	25	Mammals[a]
n-Butanol	65,100±9,300	1.89±0.66	14	10,800	Tadpole[b]
n-Hexanol	2,350±200	2.23±0.40	27	570	Tadpole[b]
n-Octanol	41.5±2.7	1.56±0.20	15	57	Tadpole[b]
n-Decanol	9.34±0.86	1.86±0.32	15	12.6	Tadpole[b]
n-Dodecanol	2.79±0.34	1.85±0.46	22	4.7	Tadpole[b]
n-Tridecanol	2.70±0.30	2.01±0.46	14	[c]	Tadpole[b]
n-Tetradecanol	1.82±0.19	3.08±1.09	14	[c]	Tadpole[b]

IC_{50} concentrations and Hill coefficients are given as means±s.e.means for d.f. degrees of freedom, obtained from unweighted least squares fits of data at 3 μM 5-HT to an inhibitory Hill equation (see text). (Butanol and hexanol data at potentiating concentrations were ignored in these regressions). [a]Franks & Lieb (1994); [b]Alifimoff *et al.* (1989); [c]Not anaesthetic (Alifimoff *et al.*, 1989).

know of only one previous study of the effects of volatile agents on the 5-HT$_3$ receptor. Using cloned mouse 5-HT$_3$ receptors expressed in *Xenopus* oocytes and two of the volatile anaesthetics studied here (halothane and isoflurane), Machu & Harris (1994) found larger potentiations with halothane than with isoflurane, in agreement with our results. A direct comparison can be made for the 1 MAC (310 μM) isoflurane potentiation of the response to 1 μM 5-HT, and their value of $\sim$15% is very similar to ours. It thus appears that, at clinical levels, isoflurane is anomalously ineffective at potentiating 5-HT$_3$ receptor activity, so that a unitary role for the 5-HT$_3$ receptor in the general anaesthesia produced by volatile anaesthetics seems unlikely. (A related issue, which we will not consider further, is that some of the pharmacological properties of the 5-HT$_3$ receptor appear to be species-dependent (Peters *et al.*, 1992; Miyake *et al.*, 1995). It is possible that this species variation might extend to the anaesthetic sensitivity of 5-HT$_3$ receptors, and further work is needed to address this point).

We studied the effect of methoxyflurane, the most active volatile agent, in more detail. It did not alter the reversal potential of the 5-HT (1 μM) response, and its potentiating effect was independent of membrane potential (Figure 3). This potentiation at a low level of 5-HT did not occur at a high (50 μM) 5-HT concentration (Figure 4), consistent with the results of Machu & Harris (1994) for halothane and isoflurane acting on a cloned 5-HT$_3$ receptor expressed in *Xenopus* oocytes. When studied over a range of 5-HT concentrations, methoxyflurane was found to shift dose-dependently the 5-HT dose-response curve to lower 5-HT concentrations without significantly affecting the Hill coefficient or maximum response (Figure 5). At the surgically relevant concentration of 1 MAC, methoxyflurane decreased the EC_{50} concentration for 5-HT by over 40% (Figure 5). This large shift is consistent with methoxyflurane allosterically increasing the affinity of the 5-HT$_3$ receptor for 5-HT. Similar leftward shifts of agonist dose-response curves by the volatile general anaesthetic, halothane, have been documented for potentiation of inhibitory GABA$_A$ and glycine receptors (Wakamori *et al.*, 1991), both of which are members of the same superfamily of ligand-gated receptors to which the excitatory 5-HT$_3$ receptor belongs, and it is tempting to suppose that a common mechanism might be involved.

Intravenous general anaesthetics

Thiopentone is the most widely used barbiturate agent in clinical general anaesthesia. Perhaps surprisingly in view of the above results with volatile anaesthetics, thiopentone did not potentiate but instead inhibited responses to 5-HT. The thiopentone dose-response curve at 1 μM 5-HT (see Figure 6) exhibited a high IC_{50} concentration (115$\pm$10 μM) compared with the EC_{50} for general anaesthesia ($\sim$25 μM; Franks & Lieb, 1994) and a Hill coefficient (1.7$\pm$0.3) substantially greater than unity. The inhibition was the same at 5-HT concentrations both above and below the 5-HT EC_{50}, suggesting a non-competitive mode of inhibition, while the size of the Hill coefficient suggests multiple sites of barbiturate binding. The fact that the IC_{50} concentration is almost five fold the EC_{50} for general anaesthesia, with only $\sim$10% inhibition occurring at the EC_{50}, suggests that inhibition of the 5-HT$_3$ receptor by thiopentone is not a major factor in either the induction of general anaesthesia or the production of unwanted side-effects.

We know of only one previous study on the effects of thiopentone on the 5-HT$_3$ receptor. Barann *et al.* (1993) looked at the effects of a large number of drugs on the 2 min uptake of [^{14}C]-guanidinium by N1E-115 neuroblastoma cells induced by the continuous application of 100 μM 5-HT. This protocol has little resemblance to ours (for example, the 5-HT concentration was over 50 fold above our EC_{50}), and the IC_{50} obtained (71$\pm$13 μM thiopentone) was significantly less than ours.

Not all intravenous general anaesthetics inhibit the 5-HT$_3$ receptor. This is clear from a study with ketamine by Peters *et al.* (1991) on rabbit nodose ganglion neurones, which showed dose-related enhancements of peak 5-HT responses over the range 3–30 μM ketamine. (However, the effect was only $\sim$10% at 3 μM ketamine). Propofol (1.1–22 μM), on the other hand, has been shown to have no significant effect on cloned 5-HT$_3$ receptors expressed in *Xenopus* oocytes (Machu & Harris, 1994). Overall, therefore, the few reliable studies there have been on the 5-HT$_3$ receptor using intravenous general anaesthetics do not support a simple unitary molecular mechanism. Moreover, it would appear that the varied effects observed are all very small at EC_{50} concentrations for mammalian general anaesthesia (Franks & Lieb, 1994).

The n-*alcohols*

While it is well-known (Lovinger, 1991; Lovinger & White, 1991; Lovinger & Zhou, 1994; Machu & Harris, 1994; Downie *et al.*, 1995) that the lower *n*-alcohols (ethanol in particular) can potentiate 5-HT$_3$ receptor responses to low levels of 5-HT, we are aware of no work that has been published on the effects of the higher *n*-alcohols (from hexanol upwards). This represents a substantial gap in the literature, since, although these agents are not important clinically, there exists a large literature on the effects of the *n*-alcohols on general anaesthesia in aquatic animals, as well as on ion channels and lipid and enzyme models of general anaesthesia (see, for example, Franks & Lieb, 1985; Alifimoff *et al.*, 1989; Miller *et al.*, 1989; McKenzie *et al.*, 1995).

Our principal findings from the present studies were (i) that all of the *n*-alcohols tested (from butanol to pentadecanol) were able to inhibit the 5-HT$_3$ receptor (see Figure 7) and (ii) that the *n*-alcohols exhibit a cut-off in inhibitory potency similar to that found for tadpole general anaesthesia. The *n*-alcohol IC_{50} concentrations and Hill coefficients for inhibiting the 5-HT$_3$ receptor are listed in Table 1, together with EC_{50} concentrations for general anaesthesia. It can be seen that the Hill coefficients are greater than unity (suggesting multiple alcohol-binding sites) but are not statistically different from each other (so that IC_{50} concentrations can be meaningfully compared). It can also be seen from Table 1 that the IC_{50} concentrations for inhibiting the 5-HT$_3$ receptor are substantially greater than the general anaesthesia EC_{50} concentrations for the lower alcohols butanol and hexanol (presumably due to some residual potentiation), whereas the values are quite similar for the anaesthetic *n*-alcohols octanol, decanol, and dodecanol. This suggests inhibitory alcohol-binding pockets or clefts on the 5-HT$_3$ receptor with sizes comparable to those found on the putative proteins responsible for alcohol general anaesthesia in tadpoles.

This can perhaps be better visualised by looking at Figure 8, where we have plotted together the IC_{50} and EC_{50} concentrations of the *n*-alcohols, together with their maximum aqueous solubilities (c_{sat}), against the number of carbon atoms in the alcohols. Figure 8 shows that IC_{50} concentrations decrease with alcohol size (that is, potency increases) up to and including dodecanol, at which point the IC_{50} concentrations are similar for dodecanol, tridecanol and tetradecanol. While the IC_{50} concentrations plateau out at this point, however, the aqueous solubilities continue to decrease until a 'cutoff' point is reached at tetradecanol. Here the IC_{50} concentration is greater than the maximum aqueous solubility and is thus experimentally unobtainable. We have interpreted such 'cutoffs' in the general anaesthesia data, as well as in luciferase enzymes, as being ultimately due to the alcohol obtaining a size greater than the size of the alcohol-binding pockets or clefts on the relevant proteins (Franks & Lieb, 1985; Curry *et al.*, 1990; Moss *et al.*, 1991). Here it appears that the inhibitory alcohol-binding sites on the 5-HT$_3$ receptor have sizes roughly equal to that for dodecanol, so that larger *n*-alcohols have roughly constant IC_{50} values. A prediction of this hypothesis is that the IC_{50} for pentadecanol should be far above its aqueous solubility and thus even a saturated solution of pentadecanol

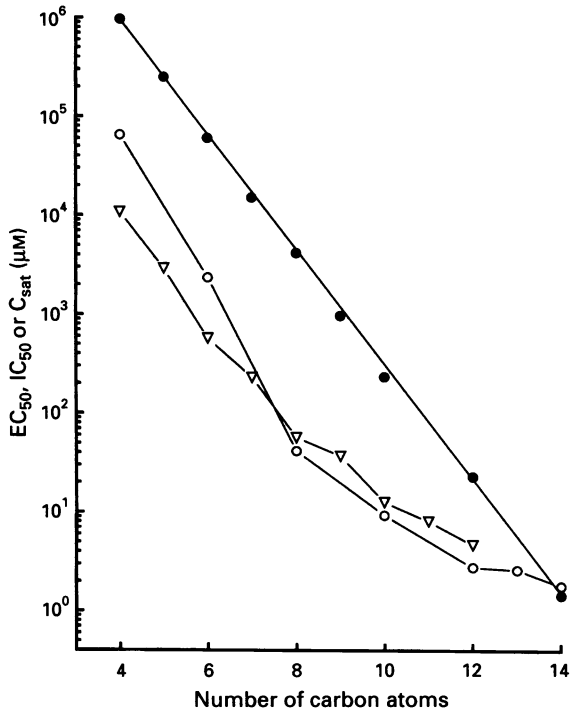

Figure 8 The cutoff effect for the *n*-alcohols. IC_{50} concentrations for inhibiting the 5-HT_3 receptor channel (○), EC_{50} values for tadpole general anaesthesia (▽) and aqueous solubilities c_{sat} (●) are plotted on a logarithmic scale against the number of carbon atoms in each *n*-alcohol. The IC_{50} and EC_{50} concentrations are from Table 1, while values for the aqueous solubilities, together with the straight line, are from Bell (1973) and sources referenced therein.

should not be able to inhibit substantially the 5-HT_3 receptor. Indeed, we found that a 98% saturated solution of *n*-pentadecanol inhibited the receptor by only 15%.

Our results (see Figure 7) with the *n*-alcohols suggest the existence on the 5-HT_3 receptor of two classes of alcohol-binding sites: one for potentiation and another for inhibition. Since inhibition is found for all alcohols from butanol to pentadecanol, whilst potentiation is found only for the lower alcohols, it is tempting to suppose that the inhibitory sites are larger in size (or possibly more apolar in nature) than the potentiating sites. Furthermore, since the activity of the potentiating sites seems to disappear after hexanol, while that of the inhibitory sites plateaus off after dodecanol, our data suggest that the inhibitory sites might be roughly twice as large as the potentiating sites. However, much more quantitative data, especially at low concentrations of 5-HT, are required before this hypothesis can be properly tested.

Comparison with anaesthetic effects on other members of the superfamily

It is interesting to compare the effects of general anaesthetics that we have observed on the 5-HT_3 receptor with those that have been observed on the other members of the superfamily of ligand-gated receptor channels (nicotinic ACh, $GABA_A$ and glycine receptors) to which it belongs. Our results show that 5-HT_3 receptor activity is potentiated by volatile anaesthetics (Figure 2) and low levels of the lower *n*-alcohols (Figure 7) but is inhibited by a barbiturate (Figure 6) and the higher *n*-alcohols (Figure 7).

Qualitatively similar behaviours are found with other members of the superfamily. For example, volatile anaesthetics potentiate both $GABA_A$ and glycine receptor activities (Nakahiro *et al.*, 1989; Wakamori *et al.*, 1991; Harrison *et al.*, 1993; Hall *et al.*, 1994), while barbiturates inhibit the activities of both neuronal (Pocock & Richards, 1987) and muscle-type (de Armendi *et al.*, 1993) nicotinic ACh receptors. Higher alcohols also inhibit both neuronal (McKenzie *et al.*, 1995) and muscle-type (Wood *et al.*, 1991) nicotinic ACh receptors. Finally, a separate, potentiating site for short-chain alcohols is a feature of muscle-type nicotinic ACh receptors (Wood *et al.*, 1991). On the other hand, the 5-HT_3 receptor does not possess some properties belonging to certain members of the superfamily, such as the potentiation by barbiturates seen with the $GABA_A$ receptor (Parker *et al.*, 1986) and the inhibition by volatile agents observed with the neuronal nicotinic ACh receptor (McKenzie *et al.*, 1995). Nonetheless, for what is thought to be a primitive member of the superfamily (Ortells & Lunt, 1995), the 5-HT_3 receptor possesses a surprisingly rich repertoire of potentiating and inhibitory sites for general anaesthetics.

Implications for general anaesthesia

We have found that, at EC_{50} concentrations for general anaesthesia, different anaesthetic agents can affect the activity of the 5-HT_3 receptor in qualitatively and quantitatively different manners. Methoxyflurane, enflurane and halothane exerted strong potentiating effects, while the higher *n*-alcohols (from octanol to dodecanol) produced large inhibitions. Other agents (isoflurane, thiopentone, butanol and hexanol) produced much smaller effects at these concentrations, as has been previously reported for propofol (Machu & Harris, 1994). Overall, then, at EC_{50} concentrations for general anaesthesia, some agents strongly potentiate, others strongly inhibit, while still others have little effect upon 5-HT_3 receptor function.

This behaviour is clearly inconsistent with any simple unitary theory of general anaesthesia involving effects on 5-HT_3 receptors but does not rule out 5-HT_3 receptor contributions to the states of general anaesthesia produced by the more active anaesthetic agents. While it is easy to accept that anaesthetic inhibition of the excitatory 5-HT_3 receptor might positively contribute to general anaesthesia, it is perhaps less obvious how anaesthetic potentiation of this receptor might so contribute. However, it has been found that 5-HT acting on 5-HT_3 receptors in the rat hippocampus can excite inhibitory GABAergic interneurones (Ropert & Guy, 1991), so that anaesthetic potentiation of 5-HT_3 receptors could increase overall inhibition in the (still unknown) CNS regions mediating general anaesthesia. Nonetheless, any satisfactory interpretation of how the effects we have observed *in vitro* might translate into the *in vivo* situation will require more definitive evidence on the relative extent to which 5-HT_3 receptors are involved in 'background' neuromodulation or fast synaptic transmission in the CNS.

As regards anaesthetic side-effects, there is much current interest in the role of 5-HT_3 receptors in postoperative nausea and vomiting (PONV). Since PONV can be reduced by selective 5-HT_3 antagonists (Haigh *et al.*, 1993), one might suppose that anaesthetics which inhibit or minimally potentiate the 5-HT_3 receptor might cause less PONV than agents which substantially enhance 5-HT_3 activity. Indeed, it does seem true that propofol, which affects the 5-HT_3 receptor minimally (Machu & Harris, 1994), is associated with a relatively low incidence of PONV (Rabey & Smith, 1992). On the other hand, data from outcome studies suggest that there are no significant differences between halothane, enflurane and isoflurane with respect to the incidence of postoperative emesis (Watcha & White, 1992), even though there is greater potentiation of the 5-HT_3 receptor with halothane and enflurane compared with isoflurane (see Figure 2). We feel, therefore, that it is premature to decide on the basis of the limited data available whether or not anaesthetic effects on the 5-HT_3 receptor play an important role in producing PONV. More carefully controlled studies are needed to establish the true clinical situation (Rabey & Smith, 1992; Watcha & White, 1992), and the effects of a larger number of general anaesthetics on the 5-HT_3 receptor must be determined.

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