Differential Sensitivities of Mammalian Neuronal and Muscle Nicotinic Acetylcholine Receptors to General Anesthetics

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Background: Nicotinic acetylcholine receptors (nAChRs) are members of a superfamily of fast neurotransmitter-gated receptor channels that includes the γ-aminobutyric acid$_A$ (GABA$_A$), glycine and serotonin type 3 (5-HT$_3$) receptors. Most previous work on the interactions of general anesthetics with nAChRs has involved the muscle-type receptor. The authors investigate the effects of general anesthetics on defined mammalian neuronal and muscle nAChRs expressed in *Xenopus* oocytes.

Methods: Complementary deoxyribonucleic acid (cDNA) or messenger ribonucleic acid (mRNA) encoding for various neuronal or muscle nAChR subunits was injected into *Xenopus* oocytes, and the resulting ACh-activated currents were studied using the two-electrode voltage-clamp technique. The effects of halothane, isoflurane, sevoflurane, and propofol on the peak acetylcholine-induced currents were investigated, and concentration–response curves were constructed.

Results: The neuronal nAChRs were found to be much more sensitive to general anesthetics than were the muscle nAChRs, with IC$_{50}$ concentrations being 10- to 35-fold less for the neuronal receptors. For the inhalational general anesthetics, the IC$_{50}$ concentrations were considerably less than the free aqueous concentrations that cause general anesthesia in mammals. In addition, qualitative (dependence on acetylcholine concentration) and quantitative (steepness of concentration–response curves) differences in the anesthetic interactions between the neuronal and muscle nAChRs suggest that different mechanisms of inhibition may be involved.

Conclusions: Although there is considerable uncertainty about the physiologic roles that neuronal nAChRs play in the central nervous system, their extraordinary sensitivity to general anesthetics, particularly the inhalational agents, suggests they may mediate some of the effects of general anesthetics at surgical, or even subanesthetic, concentrations. (Key words: Anesthetics, intravenous: propofol. Anesthetics, volatile: halothane; isoflurane; sevoflurane. Receptors, acetylcholine: muscle; neuronal nicotinic; recombinant; *Xenopus* oocytes.)

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NICOTINIC acetylcholine receptor channels (nAChRs) are members of an important superfamily of genetically and structurally related fast neurotransmitter-gated ion channels that also includes the γ-aminobutyric acid$_A$ (GABA$_A$), glycine, and serotonin type 3 (5-HT$_3$) receptor channels.[1] Neuronal nAChRs[2-7] are widely distributed in the brain and also are found in the spinal cord and peripheral nervous system, but their physiologic roles in the brain are uncertain.[8] Although other members of this superfamily (most notably GABA$_A$ receptors) almost certainly play a more crucial role in central synaptic transmission, nAChRs as a class probably have been the most intensively studied. In large part, this is because of the relative accessibility of the muscle nAChR and the closely related nAChR from the *Torpedo* electric organ. However, during the past few years, a growing number of neuronal subunits have been cloned (11 to date, $\alpha 2$-$\alpha 9$ and $\beta 2$-$\beta 4$). Consequently, emphasis has shifted toward the characterization of neuronal nAChRs expressed in neurons and recombinant expression sys-

tems, such as *Xenopus* oocytes. What has emerged from these studies is that, although structurally highly homologous to their muscle counterparts, the neuronal receptors differ in a number of important respects. For example, although all nAChRs function as pentamers, the muscle-type receptors have invariant subunit stoichiometries ($2\alpha\beta\gamma\delta$ or $2\alpha\beta\epsilon\delta$), but the neuronal receptors display a bewildering diversity of α/β heteromers and α homomers. In addition, the different subunit combinations often differ in their agonist and antagonist sensitivities, single channel properties, and rates of agonist-induced desensitization.[2,3,6,7] One simplifying feature is that when neuronal α and β subunits form heteromeric receptor channels, they appear to have a stoichiometry of two α subunits to three β subunits.[9,10] Whether this will turn out to be a universal rule remains to be seen. The various subunits have a complex pattern of expression within the central nervous system (CNS),[11–13] with the $\alpha_4\beta_2$ combination being one of the most prevalent in the brain.[14]

In parallel with studies on the structure and function of nAChRs, work with general anesthetics at the molecular level has focused almost exclusively on the muscle nAChR and that from the *Torpedo* electric organ. Many different techniques have been used, including equilibrium binding,[15–17] rapid-flux measurements,[18] patch-clamping,[19–22] and site-directed mutagenesis.[23] Although there has been some work on the effects of general anesthetics on neuronal receptors from molluscan neurones[24–26] and bovine adrenal chromaffin cells[27,28] and although there has been a preliminary report on anesthetic inhibition of recombinant neuronal receptors,[29] there have been no published studies on the interactions between general anesthetics and defined neuronal nAChRs in which the sensitivities to general anesthetics of neuronal and muscle-type receptors can be directly compared. In this article, we report our first results on the effects of three volatile general anesthetics (halothane, isoflurane, and sevoflurane) and an intravenous agent (propofol) on defined muscle and neuronal nAChRs expressed in the *Xenopus* oocyte recombinant expression system.

Materials and Methods

Preparation and Injection of Xenopus Oocytes

All experimental procedures involving *Xenopus laevis* frogs were in compliance with UK regulations. Adult female frogs (Blades Biological, Cowden, Kent,

UK) were maintained in fresh-water holding tanks at 20–22°C, with a 12-h light/12-h dark cycle. The frogs were anesthetized by immersion in a 0.2% (weight-to-volume ratio) solution of tricaine (3-aminobenzoic acid ethyl ester, methanesulfonate salt), and portions of the ovaries were surgically removed and teased apart with forceps. These portions were briefly washed in "calcium-free" oocyte incubation buffer (calcium-free OIB; composition in mM: NaCl, 88; KCl, 1; NaHCO$_3$, 2.4; MgSO$_4$, 0.8; HEPES, 15; titrated to pH 7.5 with NaOH) before incubation in the same saline containing collagenase (2 mg/ml of type 1A collagenase, Sigma Chemical Co., Poole, Dorset, UK) for 3 h at room temperature with constant agitation. After careful washing in calcium-free OIB to remove all traces of collagenase, the oocytes were transferred into normal OIB (composition in mM: NaCl, 88; KCl, 1; NaHCO$_3$, 2.4; MgSO$_4$, 0.8; CaCl$_2$, 0.4; Ca(NO$_3$)$_2$, 0.3; HEPES, 15; titrated to pH 7.5 with NaOH). Oocytes at stages 5 or 6 of development were then chosen for injection by visual inspection. Selected oocytes were injected with 10 nl of diethyl pyrocarbonate-treated water containing 0.1–1.0 pg of complementary deoxyribonucleic acid (cDNA) directly into the nucleus of the oocyte. For messenger ribonucleic acid (mRNA) injections, into the cytoplasm, 50 nl of diethyl pyrocarbonate-treated water that contained 10–40 ng of mRNA was used. Injections were carried out with a calibrated micropipette (10- to 16-μm tip diameter) and a Picospritzer II valve (General Valve Corp., Fairfield, NJ), which provided short pressure pulses of nitrogen gas. Injected oocytes were maintained in a cooled incubator (BDH, Poole, Dorset, UK) at 19 or 20°C in normal OIB containing antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml; Life Technologies, Paisley, Scotland, UK) in individual wells (200 μl per well) of 96-well microtiter plates (Life Technologies) for 2–7 days before use. Using these procedures, approximately 90% of the injected oocytes were viable and typically had resting potentials of −40 to −90 mV. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co.

Rat neuronal nAChR cDNA for the $\alpha2$, $\alpha3$, $\alpha4$, $\beta2$, and $\beta4$ subunits was kindly supplied by Jim Patrick (Baylor College of Medicine, Houston, TX) in the pSM vector, and mouse muscle nAChR cDNA for the α, β, γ, and δ subunits was kindly supplied by Jim Boulter (Salk Institute, San Diego, CA) in either the pSP64 or pSP65 vector. The pSM vector was used for nuclear injections, and the pSP64 and pSP65 vectors were used to produce mRNA for cytoplasmic injection. So that

mRNA coding for neuronal subunits could be expressed, the neuronal $\alpha 2$, $\alpha 4$, $\beta 2$, and $\beta 4$ subunits were subcloned into a modified pcDNAI/Amp vector (Invitrogen, Leek, The Netherlands), which allowed mRNA transcription under a T7 promoter. For all injections, whether nuclear or cytoplasmic, equal amounts of either cDNA or mRNA, respectively, were used for each of the chosen receptor subunits.

Recording Technique for Xenopus Oocytes

Oocytes were placed in a bath (volume ≈ 50 μl) and continuously perfused at ~ 2 ml/min with either control or test solutions. Ionic currents evoked by bath application of acetylcholine chloride (0.3 μM – 5 mM) were recorded using the two-electrode voltage-clamp technique with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Electrodes were fabricated from thin-walled filamented borosilicate glass capillary tubes (GC150TF, Clark Electromedical Instruments, Reading, Berkshire, UK) using a two-stage pull (David Kopf Instruments vertical pipette puller, Model 720, Tujunga, CA). Electrodes were filled with 2.5 M KCl and had typical resistances of 0.4 – 0.8 $M\Omega$; the current-passing electrode usually also contained 100 mM BAPTA. Currents were filtered at 5 Hz (−3 dB, 8-pole Bessel filter; Frequency Devices, Model 902, Haverhill, MA) before being digitized (at 20 Hz) and stored on a computer. The saline used for electrophysiologic recordings had the following composition (mM): NaCl, 110; KCl, 2; $MgCl_2$, 1; $BaCl_2$, 2; and HEPES, 10 (titrated to pH 7.6 with NaOH). In addition, in almost all experiments, 100 nM atropine was used, although this did not affect the anesthetic sensitivity ($P > 0.4$). Acetylcholine solutions were prepared on the day of the experiment. In some preliminary experiments, the $BaCl_2$ was replaced by $CaCl_2$; this also did not alter the anesthetic sensitivity ($P > 0.3$). Experiments were performed at room temperature (21 – 23°C).

Acetylcholine was applied (typically for 20 – 30 s) until a clear maximum in the response was observed. The peak current was taken as a measure of receptor activity. When constructing acetylcholine concentration – response curves, the data were normalized to a standard acetylcholine concentration, which was applied alternately throughout the experiment to correct for any ''run down'' or ''run up'' in the current. In our preliminary experiments, we found that in the presence of anesthetics, successive acetylcholine responses took 1 or 2 min to achieve a steady-state value. We had previously observed a similar behavior with the inhibition

by long-chain alcohols of a neuronal nAChR from molluscan neurons,[26] and we have subsequently found (R. Dickinson, unpublished observation) that the time-course for anesthetic inhibition to achieve a steady-state value appears to depend on the acetylcholine concentration. Consequently, anesthetics were always preapplied for 2 or 3 min before the coapplication of acetylcholine. Once again, acetylcholine was applied until a clear peak was observed and repeatedly applied until a consistent response was obtained. The anesthetics on their own usually (>95% of the oocytes) had no significant effect on the resting current, but even when they did, their preapplication established an accurate baseline. In almost all cases, except at the highest concentrations of propofol (>50 μM), anesthetic inhibition was reversible, and the percentage inhibition was calculated by averaging the control responses before and after anesthetic application and averaging at least two current responses in the presence of anesthetic. In those few cases when irreversibility was seen, we ignored the subsequent control responses.

Data Analysis

Acetylcholine concentration – response data were fitted (unweighted least-squares) to a Hill equation of the form

$$E = \frac{100A^{n_H}}{A^{n_H} + (EC_{50})^{n_H}} \quad (1)$$

where E is the peak acetylcholine-induced current expressed as a percentage of the maximal current; A is the acetylcholine concentration; n_H is the Hill coefficient, and EC_{50} is the acetylcholine concentration for a half-maximal effect.

Anesthetic inhibition data were fitted (unweighted least-squares) to a Hill equation of the form

$$y = \frac{100(IC_{50})^{n_H}}{I^{n_H} + (IC_{50})^{n_H}} \quad (2)$$

where y is the percentage of the control peak acetylcholine current remaining in the presence of an anesthetic concentration I; n_H is the Hill coefficient, and IC_{50} is the anesthetic concentration for 50% inhibition.

Values throughout the paper are given as means $\pm$ SEMs. Statistical significance was assessed using Student's t test.[30]

Preparation and Delivery of Anesthetic Solutions

The volatile anesthetics were made up as fractions of saturated aqueous solutions at room temperature. The

Table 1. Percentage Inhibition by 310 μM ($\sim$1, minimum alveolar concentration [MAC]) Isoflurane of Acetylcholine-induced Currents for Various Combinations of Neuronal Nicotinic Acetylcholine Receptor Subunits

	α_2	α_3	α_4
β_2	88 $\pm$ 2.4 (n = 5)	84 $\pm$ 0.8 (n = 6)	88 $\pm$ 0.8 (n = 5)
β_4	75 $\pm$ 1.6 (n = 10)	73 $\pm$ 1.9 (n = 7)	72 $\pm$ 1.4 (n = 9)

The ACh concentration was 1 μM. The errors are SEMs for n oocytes.

saturated solutions were prepared by adding $\sim$0.2 g of the liquid anesthetic to $\sim$20 ml of buffer in a tightly sealed glass scintillation vial with a minimal air space. The vial was then vigorously shaken for 5 min before centrifuging at $\sim$800 $\times$ g for 10 min at room temperature. The concentrations of the saturated solutions were taken to be 15.3 mM isoflurane,[25] 17.5 mM halothane,[31] and 11.8 mM sevoflurane.[32] Glass reservoirs containing volatile anesthetics were sealed with a rigid plastic float, and all tubing and valves were made of polytetrafluoroethylene (PTFE). With these precautions, losses of volatile agents from the perfusion system were found to be negligible ($<$5%) when measured by gas chromatography.[33] The sources of the anesthetics were as follows: isoflurane and sevoflurane (Abbott Laboratories Ltd., Queenborough, Kent, UK); halothane (ICI Ltd., Macclesfield, Cheshire, UK). Halothane was used as supplied and contained 0.01% thymol. Propofol in its pure form (*i.e.*, 2,6-di-isopropylphenol without Intralipid®) was kindly supplied by Zeneca (Macclesfield, Cheshire, UK). Propofol stock solutions were made up in ethanol. The final concentration of ethanol in propofol-containing solutions was 17 mM. For these experiments, 17 mM ethanol was also added to the control solutions.

Results

In our first experiments, we found that all of the neuronal nAChR subunit combinations we tested were surprisingly sensitive to isoflurane. At 310 μM ($\sim$1 MAC for the rat[34]) isoflurane, the neuronal receptors were inhibited by 70–90% (table 1). Moreover, those combinations that contained the β_2 subunit appeared to be significantly more sensitive ($P < 0.01$) than those containing the β_4 subunit. These preliminary experiments were carried out at a single, very low nondesensitizing concentration of acetylcholine (1 μM). So that a fair comparison could be made of the anesthetic sensitivities of the different subunit combinations (whose sensi-

tivities may depend on the acetylcholine concentration), we determined the acetylcholine EC_{50} concentrations for selected subunit combinations so that anesthetics could be applied to receptors that were in roughly equivalent functional states. All of the acetylcholine receptors behaved in a qualitatively similar fashion in their responses to acetylcholine; low concentrations of the agonist induced small, nondesensitizing currents, whereas high concentrations of acetylcholine induced large and relatively rapidly inactivating currents (see insets to fig. 1). The neuronal receptors, however, were much less sensitive to acetylcholine than the muscle receptor, with EC_{50} concentrations roughly an order of magnitude higher (fig. 1 and table 2). For all receptors, the Hill coefficients were close to unity.

Figure 2 shows the sensitivity of a neuronal nAChR ($\alpha_4\beta_2$) to halothane compared with the relative insensitivity of the muscle receptor to a halothane concentration 10 times higher. The IC_{50} concentrations for halo-
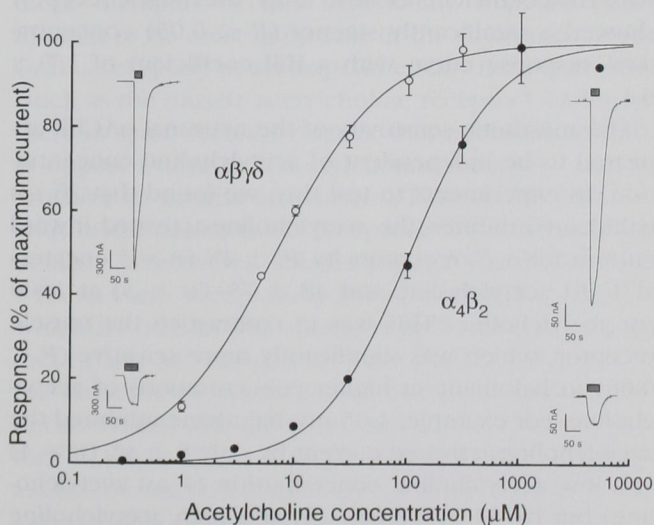

Fig. 1. Acetylcholine concentration–response curves. Data from oocytes expressing muscle receptors ($\alpha\beta\gamma\delta$ subunits; ○) and neuronal receptors ($\alpha_4\beta_2$ subunits; ●). The data points give the mean peak currents expressed as a percentage of the maximum current, and the error bars are SEMs (for at least three oocytes). Where not shown, the error was smaller than the size of the symbol. The lines are unweighted least squares fits of the mean peak currents to a Hill equation (Equation 1, Materials and Methods section). The insets on the left are typical current traces from an oocyte expressing muscle receptors ($\alpha\beta\gamma\delta$ subunits) showing responses to a high (100 μM acetylcholine, *top left*) and a low (1 μM acetylcholine, *bottom left*) concentration of acetylcholine. The insets on the right are typical traces from an oocyte expressing neuronal receptors ($\alpha_4\beta_2$ subunits) at a high (1000 μM acetylcholine, *top right*) and a low (30 μM acetylcholine, *bottom right*) concentration of acetylcholine. Oocytes were voltage-clamped at -60 mV.

Table 2. Acetylcholine Activation and Halothane Inhibition of Selected Combinations of Nicotinic Acetylcholine Receptor Subunits

Subunit Combination	Acetylcholine Activation		Halothane Inhibition	
	EC_{50} (μM)	n_H	IC_{50} (μM)	n_H
$\alpha_2\beta_4$ (neuronal)	91 ± 11	1.0 ± 0.1	47 ± 5	1.0 ± 0.1
$\alpha_4\beta_2$ (neuronal)	104 ± 13	1.1 ± 0.1	27 ± 5	0.9 ± 0.1
$\alpha_4\beta_4$ (neuronal)	45 ± 7	0.9 ± 0.1	105 ± 17	1.0 ± 0.2
$\alpha\beta\gamma\delta$ (muscle)	6.7 ± 0.6	0.9 ± 0.1	870 ± 50	1.7 ± 0.1

EC_{50} and IC_{50} concentrations and Hill coefficients n_H, were obtained from unweighted least squares fits of dose–response data (from at least 3 oocytes) to Hill equations (see Materials and Methods section). Halothane inhibitions were determined at an acetylcholine concentration equal, or close, to the EC_{50}. The errors are SEMs.

thane inhibition are listed in table 2. In addition to the sensitivities of the neuronal receptors, which had IC_{50} concentrations up to 35 times lower than that of the muscle receptor, there also was a clear difference in the Hill coefficients, which are a measure of the steepness of the concentration–response curves. Although the neuronal receptors were inhibited by halothane with Hill coefficients close to unity, the muscle receptor showed a significantly steeper ($P < 0.05$) concentration–response curve with a Hill coefficient of 1.7 ± 0.1.

The anesthetic sensitivity of the neuronal nAChR appeared to be independent of acetylcholine concentration. In experiments to test this, we found that 26 μM halothane inhibited the acetylcholine-activated inward current for $\alpha_4\beta_2$ receptors by 49 ± 2% ($n = 3$ oocytes) at 1 μM acetylcholine and 48 ± 7% ($n = 3$) at 1600 μM acetylcholine. This was in contrast to the muscle receptor, which was significantly more sensitive ($P < 0.05$) to halothane at higher concentrations of acetylcholine. For example, 1.05 mM halothane inhibited the acetylcholine-activated current by only 8 ± 4% ($n = 4$) at a low acetylcholine concentration (1 μM acetylcholine) but by 37 ± 6% ($n = 3$) at a high acetylcholine concentration (100 μM acetylcholine).

We determined anesthetic concentration–response curves for neuronal ($\alpha_4\beta_2$) and muscle ($\alpha\beta\gamma\delta$) receptors for four clinically important general anesthetics: halothane, isoflurane, sevoflurane, and propofol (fig. 3 and table 3). For all four anesthetics, the neuronal receptor was much more sensitive than the muscle receptor, with the concentration–response curves being generally steeper with the muscle receptor.

Discussion

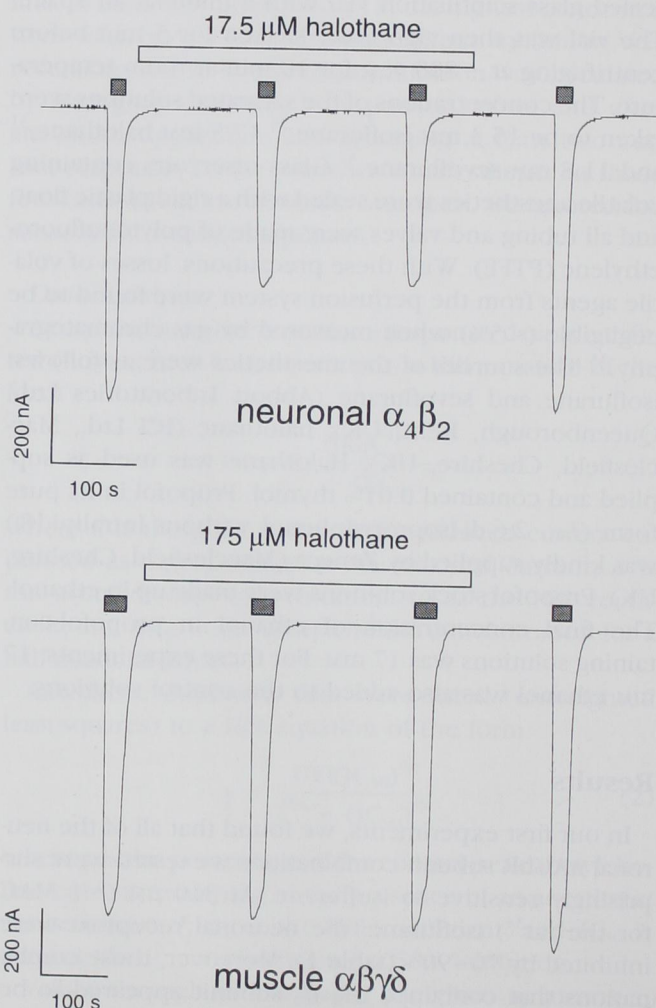Although there is no current consensus as to which molecular targets are most important in the actions of

Fig. 2. Differential sensitivity of a neuronal acetylcholine receptor ($\alpha_4\beta_2$ subunits) and the muscle receptor ($\alpha\beta\gamma\delta$ subunits) to halothane. (*Top*) Neuronal receptor. The effect of 17.5 μM halothane on acetylcholine-induced currents (160 μM acetylcholine, ■). (*Bottom*) Muscle receptor. The effect of 175 μM halothane on acetylcholine-induced currents (10 μM acetylcholine, ■). Oocytes were voltage-clamped at −60 mV.

Table 3. Differential Sensitivities of Neuronal ($\alpha_4\beta_2$) and Muscle ($\alpha\beta\gamma\delta$) Acetylcholine Receptors to Various General Anesthetics

Anesthetic	Neuronal $\alpha_4\beta_2$		Muscle $\alpha\beta\gamma\delta$		Anesthesia
	IC$_{50}$ (μM)	n_H	IC$_{50}$ (μM)	n_H	EC$_{50}$ (μM)*
Halothane	27 ± 5	0.9 ± 0.1	870 ± 50	1.7 ± 0.1	190[34]
Isoflurane	34 ± 6	0.8 ± 0.1	1160 ± 25	1.8 ± 0.1	270[34]
Sevoflurane	98 ± 8	1.1 ± 0.1	2060 ± 110	1.3 ± 0.1	300[34]
Propofol	4.5 ± 0.7	0.8 ± 0.1	46 ± 5	2.0 ± 0.5	1.5†

IC$_{50}$ concentrations and Hill coefficients, n_H, were obtained from unweighted least squares fits of dose–response data (from at least 3 oocytes) to a Hill equation (Equation 2, Materials and Methods section). The errors are SEMs. The acetylcholine concentrations were 160 μM for the neuronal nAChR and 10 μM for the muscle nAChR.

* Human free aqueous EC$_{50}$ concentration for inhibiting a purposeful response to a painful stimulus (surgical incision).

† The propofol blood concentration at which 50% of patients do not move purposefully in response to a surgical incision is 15.2 μg/ml.[52] Taking a blood/plasma partition coefficient[53] of 1.3, a value[53] for plasma protein binding of 97.8% and a molecular weight of 178 Da gives a free aqueous propofol EC$_{50}$ concentration of 1.5 μM. (This figure can only be considered to be an estimate because a small error in the value for plasma protein binding would lead to significant error in the free aqueous concentration.)

general anesthetics, there has been growing interest during the past few years in the possible role of the superfamily of fast, neurotransmitter-gated ion channels
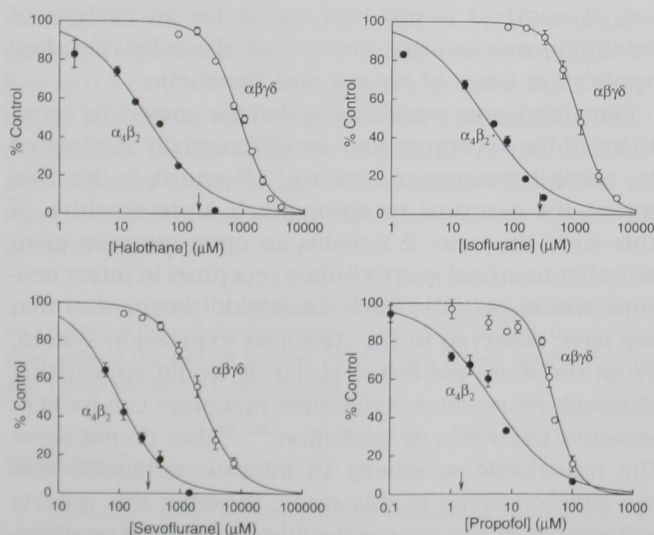

Fig. 3. Concentration–response curves showing the differential sensitivities of neuronal ($\alpha_4\beta_2$ subunits, ●) and muscle receptors ($\alpha\beta\gamma\delta$ subunits; ○) to four different anesthetics: halothane, isoflurane, sevoflurane, and propofol. The data points are the mean peak currents expressed as percentages of the control current, and the error bars are SEMs (for at least three oocytes). Where not shown, the error was smaller than the size of the symbol. The lines are unweighted least squares fits of the mean peak currents to a Hill equation (Equation 2, Materials and Methods section). The acetylcholine concentrations were 160 μM for the neuronal nAChR and 10 μM for the muscle nAChR. The vertical arrows indicate estimated human EC$_{50}$ concentrations for general anesthesia (see table 3).

that includes the GABA$_A$, glycine, nAChRs, and 5-HT$_3$ receptors.[35,36] Attention has reasonably focused on those members of this superfamily that are either thought to be most important in the CNS (such as the GABA$_A$ receptor) or are experimentally most accessible (such as the muscle acetylcholine receptor). However, there is some recent evidence that neuronal nAChRs, as opposed to muscle acetylcholine receptors, may be sensitive to general anesthetics. This has been shown with molluscan nicotinic receptors,[24-26] which are particularly sensitive to volatile general anesthetics, and with nicotinic receptors in bovine chromaffin cells,[27,28] which are inhibited by a range of different general anesthetics and are thought to have similar properties to the nicotinic receptors found in sympathetic ganglia. Recent results with glycine receptors[37] and 5-HT$_3$ receptors[38,39] show that, at least for many inhalational agents, anesthetic sensitivity may be a general feature of this superfamily of receptor channels. In contrast, the neurotransmitter-gated receptor channels activated by glutamate, the major excitatory neurotransmitter in the vertebrate CNS, have a rather different transmembrane topology[40,41] and appear to be relatively insensitive to most general anesthetics.[35,42]

The results presented here show that neuronal nAChR channels are much more sensitive to general anesthetics than their muscle counterparts under conditions where the membrane environment and intracellular milieu are identical. This seems to be true for all of the neuronal subunit combinations we have tested (see table 1), although we have concentrated on the $\alpha_4\beta_2$ combination because it is thought to be one of the most widely

expressed combinations in the brain.[14] The IC_{50} concentrations for the volatile general anesthetics halothane, isoflurane, and sevoflurane are, on average, about 30 times lower for the neuronal $\alpha_4\beta_2$ receptor than for the muscle receptor, whereas for propofol, the IC_{50} concentrations differ by an order of magnitude. Moreover, the neuronal nAChRs display a remarkable sensitivity to the volatile agents in absolute terms, with the IC_{50} for halothane inhibiting the $\alpha_4\beta_2$ combination being as low as 27 μM. This is seven times lower than the free aqueous concentration that is present at 1 MAC (see table 3).

Why are the neuronal nAChRs so much more sensitive to anesthetic inhibition? In addressing this question, one should first consider what has been learned from the numerous studies on the effects of general anesthetics on the muscle-type receptor. Probably the most definitive information has come from some of the more recent studies using patch-clamp recording from mouse muscle receptors expressed in *Xenopus* oocytes[23] and from single channel analysis of nicotinic currents in cultured cells[43-45] belonging to the muscle cell line BC3H-1. These studies present a convincing case that anesthetics such as isoflurane act predominantly by binding to a discrete site within the ion channel pore itself,[23] although the preferential binding to the open state of the channel is relative rather than absolute.[43-45] A comparison can be made between our study and that of Forman *et al.*,[23] who found with the mouse recombinant muscle receptor that 1 mM isoflurane inhibited the peak 100 μM acetylcholine-induced current by 60%, which is reasonably consistent with our observation (see table 3) of an IC_{50} concentration for isoflurane of 1.2 mM (at an acetylcholine concentration of 10 μM). Moreover, our observation that the muscle receptor is more sensitive to anesthetic inhibition at high rather than low acetylcholine concentrations (see Results) also is consistent with the proposed open channel block mechanism.[44]

In the elegant study of Forman *et al.*,[23] it was shown that anesthetics such as isoflurane probably interact with certain amino acids in the M2 domains (of the muscle receptor subunits) that line the ion channel pore. This begs the question of whether differences in the primary sequences of the neuronal and muscle subunits in this region could account for their different anesthetic sensitivities. This seems unlikely because the amino acids that would form the pore lining in a neuronal receptor (say $\alpha_4\beta_2$) are remarkably similar (not shown) to those in the muscle receptor ($\alpha\beta\gamma\delta$), with only a few conservative substitutions. Nonetheless, small differences in this region *can* result in different binding affinities for open channel blockers.[23,46] In addition, however, there are qualitative and quantitative differences between the anesthetic sensitivities of the neuronal receptors and that of the muscle receptor. For example, the concentration–response curves for anesthetic inhibition generally are less steep for the neuronal receptors (see tables 2 and 3). The inhibitory Hill coefficients are close to unity for all anesthetics and neuronal subunit combinations tested. For the muscle receptor, the Hill coefficients are larger than unity, suggesting that more than one anesthetic molecule could be involved in the inhibition. (An alternative explanation of the different Hill coefficients is that anesthetics affect channel inhibition and desensitization rates to different extents for the two subtypes.) Another difference is that inhibition of the neuronal receptor appears to be independent of agonist concentration (see Results section), suggesting a mechanism of inhibition different from the open channel block found with the muscle receptor[23,43-45]; we did not explore this finding because the technique used here (two-electrode voltage clamping of oocytes) is not best suited for an analysis of inhibition mechanisms because of the relatively slow application times of agonist and anesthetic.

One interesting possibility is that the anesthetic sensitivity of the receptors may be differentially modulated by second messenger systems,[47-49] and it is this that makes the neuronal receptor much more sensitive. If this were the case, it remains an open question as to whether neuronal acetylcholine receptors in intact neurons will display the same anesthetic sensitivities that we have observed in the *Xenopus* expression system. From the work published so far, it would appear that although neuronal acetylcholine receptors can be very sensitive to anesthetic inhibition,[24-28] they do not show the remarkable sensitivity to inhalational agents that we have observed in this study. Whether this reflects differences in the neuronal subunits involved or differences in the cellular environment remains to be seen. In this context, it is worth noting that although acetylcholine receptors expressed in oocytes often have properties that closely resemble native receptors, differences have been reported at the single channel level.[50] Future work with muscle and neuronal hybrid receptors and genetically engineered muscle and neuronal chimeric receptors should help determine the molecular basis of the anesthetic sensitivity of the neuronal receptors. Whatever the reasons, our results show that simple in-

halational general anesthetics can exert substantial effects on neuronal ion channels at far lower concentrations than previously shown. The IC_{50} concentrations for the three inhalational anesthetics are considerably lower (~0.1–0.3 MAC) than the free aqueous concentrations present during general anesthesia (see table 3). In contrast, although propofol is considerably more effective at inhibiting the neuronal receptor than the muscle receptor, its IC_{50} concentration for inhibition of the neuronal receptor is three times higher than the free aqueous EC_{50} concentration needed to inhibit a purposeful response to a painful stimulus (table 3). Nonetheless, at this EC_{50} concentration (1.5 μM), propofol inhibits the neuronal receptor by 30%.

What relevance do our results, showing that neuronal nAChRs are sensitive to inhibition by general anesthetics, have to mammalian general anesthesia? This is a difficult question to answer, particularly when the role of nAChRs in the brain is so uncertain.[8] Nonetheless, the possible importance of central nAChRs is underscored by their wide distribution within the CNS[11-13] and by the recent demonstration that they can modulate transmission across glutamatergic synapses.[51] Our results show that neuronal nAChRs can be substantially inhibited at inhalational anesthetic concentrations as low as 0.1 MAC, so these findings may have some bearing on anesthetic effects observed at subanesthetic concentrations. As the function of nAChRs in the CNS becomes better understood, it should then be possible to more accurately assess the relevance of our results to general anesthesia.

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