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Modulation of Recombination Human γ-Aminobutyric Acid$_A$ Receptors by Isoflurane

Influence of the Delta Subunit

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Background: **The γ-aminobutyric acid (GABA)$_A$ receptor/chloride channel has a broad-spectrum anesthetic sensitivity and is a key regulator of arousal. Each receptor/channel complex is an assembly of five protein subunits. Six subunit classes have been identified, each containing one to six members; many combinations are expressed throughout the brain. Benzodiazepines and intravenous anesthetic agents are clearly subunit dependent, but the literature to date suggests that volatile anesthetics are not. The physiological role of the δ subunit remains enigmatic, and it has not been examined as a determinant of anesthetic sensitivity.**

Methods: **Combinations of GABA$_A$ receptor subunit cDNAs were injected into *Xenopus laevis* oocytes: $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2L}$, $\alpha_1\beta_1\delta$, and $\alpha_1\beta_1\gamma_{2L}\delta$. Expression of functional ion channels with distinct signalling and pharmacologic properties was demonstrated within 1–4 days by established electrophysiological methods.**

Results: **Co-expression of the δ subunit produced changes in receptor affinity; current density; and the modulatory efficacy of diazepam, zinc, and lanthanum; it also produced subtle changes in the rate of desensitization in response to GABA. Isoflurane enhanced GABA-induced responses from all combinations: $\alpha\beta\delta$ (>10-fold) > $\alpha\beta$ > $\alpha\beta\gamma \geq \alpha\beta\gamma\delta$ (≈5-fold). Dose–response plots were bell shaped. Compared with $\alpha\beta\gamma$ receptors (EC_{50} = 225 μM), both $\alpha\beta\delta$ (EC_{50} = 372 μM) and $\alpha\beta\gamma\delta$ (EC_{50} = 399 μM) had a reduced affinity for isoflurane. Isoflurane (at a concentration close to the EC_{50} for each subunit) increased the affinity of GABA for its receptor but depressed the maximal response ($\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$). In contrast, the small currents through $\alpha\beta\delta$ receptors were enhanced, even at saturating agonist concentrations.**

Conclusions: **δ Subunit expression alters GABA$_A$ receptor function but is not an absolute determinant of anesthetic sensitivity. (Key words: Electrophysiology; inhalational subunit ion channels; oocytes.**

γ-AMINOBUTYRIC acid (GABA) is the most important inhibitory neurotransmitter in the mammalian brain, where it is present at up to one third of all synapses.[1] The GABA$_A$ receptor is a member of the ligand-gated ion channel superfamily[2] composed of an aggregation of five subunits[3] around an integral fast chloride channel. Six classes (α, β, γ, δ, ρ, and ϵ) of GABA receptor subunits have been cloned to date, with as many as six distinct proteins in each. This allows for many different combinations and physiologic or pharmacologic isomerism.[4,5] Subunits are expressed differentially throughout the central nervous system.

Anesthetics exert their actions on the central nervous system by modulating neuronal excitability. At least three mechanisms have been proposed to account for such depressant effects: (1) Anesthetics dissolve in the bulk lipid matrix of the biologic membrane to alter fluidity or volume[6]; (2) The molecules interact directly with hydrophobic binding sites on signalling proteins within the plasma membrane[7]; or (3) Target proteins are affected indirectly by second messenger systems.[8] Members of the ligand-gated ion channel superfamily are now recognized as stereoselective targets for these drugs and are modulated by clinically relevant concentrations (*e.g.*, refs. 7 and 9). Several laboratories are currently attempting to shed light on the submolecular identity and location of domains conferring such sensitivity on ligand-[10,11] or voltage-gated channels.[12]

A broad range of drugs with widely differing structures have been shown to act at or on the GABA$_A$ receptor, including sedative, hypnotic, anticonvulsant, and anesthetic agents (reviewed in ref. 13). Benzodiazepine agonists

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only produce their characteristic potentiating effect if a γ subunit is co-expressed with α and β.[14] The α subunit has been seen to subtly alter sensitivity to benzodiazepines[15] and to steroids.[16] Sedative concentrations of ethanol (5–30 mM) require the γ subunit and, more specifically, the extra eight amino acids in the longer splice variant of the γ subunit (γ_{2L}), for receptor sensitivity.[17] In the early 1990s, several groups reported the enhancement of $GABA_A$ currents by inhalational anesthetics.[18–20] Isoflurane[21] and enflurane[22] appeared to cause an increase independently of the presence of α, β, or γ, although the latter study did suggest that there was a qualitative difference in modulation due to subunit composition. Also, pentobarbital and propofol action have been shown to be markedly dependent on the presence of the newly identified ϵ subunit.[5] Five ρ subunits alone (a homooligomeric pentamer) can form a functional receptor complex; to date this is the only chloride channel activated by GABA that is either completely insensitive[21] or depressed by volatile anesthetics.[23] The α_6 subunit strongly increases the affinity and efficacy of direct activation of recombinant human $GABA_A$ receptors by pentobarbital (in the absence of neurotransmitter).[11] The subunit dependence of anesthetic action is topical and has been comprehensively reviewed.[24]

The δ subunit was cloned relatively recently,[25] and distribution of the rat δ mRNA has been mapped using *in situ* hybridization studies. The most prominent expression was noted in the cerebellum, with significant levels in the thalamic nuclei, dentate gyrus, olfactory bulb and tubercle, cerebral cortex, and nucleus accumbens.[26] Immunohistochemistry broadly confirms this distribution pattern, and immunoprecipitation experiments suggest that ≈21% of solubilized rat brain receptors contain the δ subunit.[27] Despite this widespread distribution and the pivotal role of $GABA_A$ receptors in neuronal inhibition, few articles on physiologic signalling roles for δ have been published (*e.g.,* ref. 28). Rat brain isoforms were characterized in some detail, but modulation by pentobarbital was not notably dependent on co-expression of δ subunits.[29] The aims of the current study were to seek evidence for the functional expression of δ in recombinant $GABA_A$ receptors and then to examine the influence of this subunit on modulation by isoflurane.

Materials and Methods

Oocyte Preparation

Adult female *Xenopus laevis* (imported from African Xenopus Facility, Noordhoek, South Africa) were kept in a standard glass tank with a 12:12-h light:dark cycle. The water was recirculated through a filter and thermostatically controlled at ≈23°C. Feeding was carried out daily and consisted of standard *Xenopus* pellets. Donor frogs were anesthetized by immersion in 2–3 cm of 0.4% 3-aminobenzoic acid ethyl ester (tricaine) before oophorectomy using standard surgical techniques. Viable donor frogs were not reused within 6 weeks of this procedure. Ovarian lobes were cut and collected into calcium-free saline (OR2), which contained 82 mM of NaCl, 2 mM of KCl, 5 mM of HEPES, and 1 mM of $MgCl_2$, titrated to pH 7.5 with NaOH. The lobes were washed in a culture dish using OR2 before immersion in a thawed aliquot (3 ml) of collagenase buffer (2 mg/ml of OR2) and digested for 8–10 min. The collagenase was removed and the oocytes were washed four or five times. Stage V and VI oocytes were manually dissected from their loosened epithelial and thecal layers using a low-powered microscope (12.5× + 2.5×) and fine-tipped forceps. Stripped oocytes were resoaked in collagenase for 3–5 min to remove remaining follicular cells, with the enzyme action quenched by immersion in albumin (15 mg/10 ml of OR2) for 5–10 min. Finally, cells were transferred into filtered (0.2 μm; Gelman Sciences, Ann Arbor, MI) Modified Barth's saline containing 88 mM of NaCl, 1 mM of KCl, 0.82 mM of $MgSO_4$, 0.33 mM of $Ca(NO_3)_2$, 0.41 mM of $CaCl_2$, 2.5 mM of $NaHCO_3$, and 10 mM of HEPES, titrated to pH 7.4 with NaOH, which had been autoclaved and supplemented with gentamicin (100 mg/l), theophylline (90 mg/l), penicillin (10,000 U/l), and streptomycin (10 mg/l).

DNA Injection

Samples of human $GABA_A$ receptor subunit cDNAs $\alpha_{1.2}$, $\beta_{1.3}$, γ_{2L} and δ were provided by Dr. Paul Whiting (M.S.D., Harlow, United Kingdom) in the pCDM8 eukaryotic expression vector. Stock concentrates of ≈1 μg/μl were made up in autoclaved water and stored at −20°C until required. For injections, 1 μl of the required subunits were added to 75 μl of a buffer consisting of 88 mM of NaCl, 1 mM of KCl, and 15 mM of HEPES, which was titrated to pH 7.0 and sterilized through 0.2-μm filters.[17] These dilutions were stored at 4°C between uses and freshly prepared every 3–4 months or when expression levels decreased.

Autoclaved micropipettes (glass capillaries; Laser, Southampton, United Kingdom) with tip sizes ranging from 10–20 μm were back-filled completely with heavy white mineral oil (Sigma Diagnostics, St. Louis, MO) and attached to the automatic Drummond microdispenser

(Laser). A 1-μl sample of the appropriate subunit combination was placed onto parafilm and drawn up into the tip of the micropipette. Oocyte nuclei were injected blindly (20 nl per oocyte) and transferred to 96-well plates containing filtered, supplemented Modified Barth's saline. Cells were incubated at 18–22°C until required (1–5 days).

Electrophysiologic Recording

Electrodes (GC150F-10; Clark Electromedical Instruments, Berkshire, United Kingdom) were back-filled (4–5 mm) with warmed 1% agar in 2 M KCl solution. When required for use, they were back-filled with liquid 2 M KCl and broken back to 0.5 and 3.0 MΩ (current and voltage electrodes, respectively). Agar bridges (bath ground) were made using 2 M of KCl and connected to the preamplifier *via* reference wells containing 2 M KCl. Oocytes were placed within a narrow perspex bath (30 μl in volume) before impalement. Cells were clamped at −70 or −40 mV, depending on the protocol, using a GeneClamp 500 Amplifier (Axon Instruments, Foster City, CA). Frog Ringer's solution, consisting of 115 mM of NaCl, 2.5 mM of KCl, 10 mM of HEPES, and 1.8 mM of $CaCl_2$ (titrated to pH 7.2 with NaOH), constantly perfused the clamped cell (10 ml/min) through 2-mm Teflon tubing by a gravity-feed mechanism. Various drug concentrations were held in 50- or 100-ml syringes and selected to flow, as required. Results were measured either as a current or as change from baseline conductance in response to a −20-mV, 200-ms voltage jump, applied at 2.5 Hz *via* a stimulus isolator (Harvard Apparatus, Kent, United Kingdom). A digital oscilloscope (RadioSpares, Corby, United Kingdom) was used to maximize clamp gain and stability. Real-time recordings were made on a Graphic 1002 Chart Recorder (Lloyd Instruments, Hampshire, United Kingdom) where traces were inadvertently inverted compared with established electrophysiologic convention. In all traces depicted, outward currents can be seen as downward deflections and inward currents as upward deflections.

Agonist GABA was applied for a duration sufficient to elicit peak responses (5–20 s). Modulatory drug responses were assessed at equilibrium (determined by repeat pulses) and expressed as a percentage of peak control GABA response (*i.e.,* percent change in input conductance). Diazepam and flumazenil were dissolved in dimethylsulfoxide (DMSO), then diluted $\geq$ 1000-fold into Ringer to yield the experimental solutions. DMSO was present at fixed levels before, during, and after the administration of these compounds. All experiments were conducted at room temperature (22–24°C).

Anesthetic Formulation and Quantitative Analysis

Saturated isoflurane solutions were diluted in Ringer's solution to the stated nominal concentration (confirmed by gas chromatography; n = 6). Isoflurane solutions were delivered from glass reservoirs covered with polyethylene (high density) floats to retard loss by evaporation and perfused through Teflon lines. To ascertain the degree of loss during preparation and handling of anesthetic solutions, gas chromatography was used with a gas-phase assay based on established methods[30] and published gas partitioning coefficients over a range of temperatures.[31] The mean (±SEM) loss of isoflurane from float-covered cylinders, over 2–3 h (the maximum duration of anesthetic experiment), was 11.3 ± 2.8% (n = 6 for each of two different anesthetic concentrations), and 87.7 ± 3.2% of the stated isoflurane concentration reached the cell up to 2.5 h after start of experimentation (n = 6). In this article, we cite nominal calculated isoflurane concentrations and consider the negligible loss only in the discussion.

Sources of Chemicals. Isoflurane was obtained from Abbott Laboratories (Kent, United Kingdom), flumazenil from Roche (Basel, Switzerland), antibiotics from Gibco/Life Technologies (Paisley, Scotland, United Kingdom), Lanthanum Chloride from BDH Laboratory Supplies (Poole, United Kingdom), and all other chemicals from Sigma Chemical Co. (Dorset, United Kingdom).

Data Analysis. Results, measured as change in baseline conductance (unless otherwise stated), were analyzed using Prism software (Graphpad; San Diego, CA) and expressed as mean ± SEM. Log dose–response curves were fit to a two-term logistic equation (minimum and maximum values fixed; concentration for 50% effect (EC_{50}) and Hill slope variable) by nonlinear regression. Statistical comparisons were done by paired, two-tailed *t* test or one-way analysis of variance (with Tukey's multiple comparison *post hoc* test), as indicated.

Results

Passive Properties

Attempts were made to express a homooligomeric receptor containing only the δ subunit, but no currents were detected in oocytes up to 5 days after injection (n = 40 from two batches of oocytes). The following

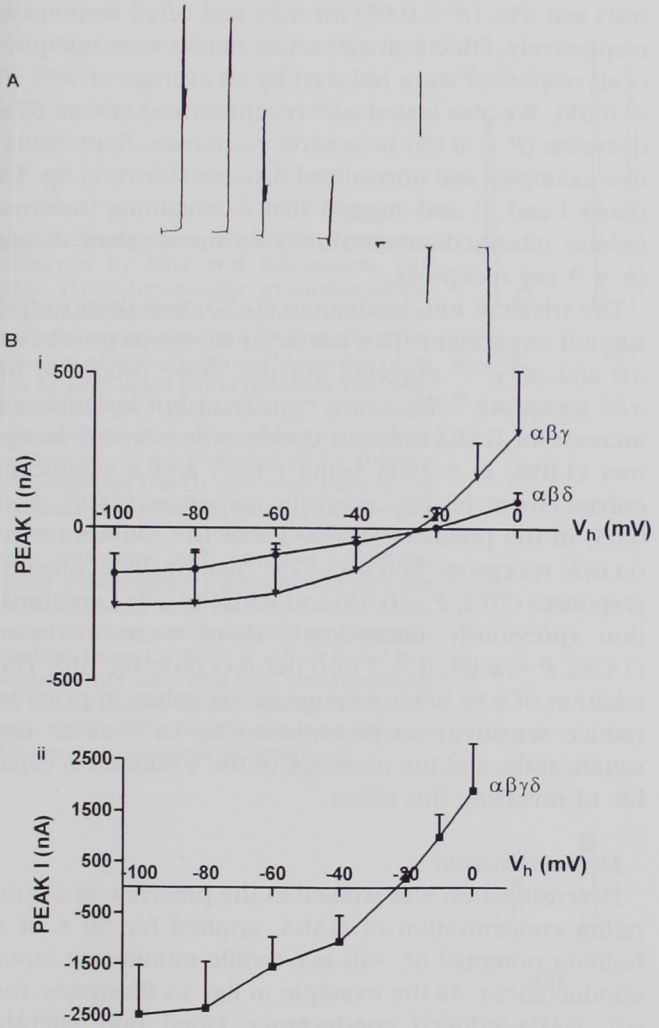

Fig. 1. ***(A)*** **Representative example of the current–voltage relationship from an oocyte injected with $\alpha\beta\delta$. Recordings were taken at the following holding potentials (from left to right): −100, −80, −60, −40, −20, −10, and 0 mV (scale bar represents 100 nA).** ***(B)*** **The compounded data (mean + SEM) are shown over the range −100–0 mV for (i) $\alpha\beta\gamma$ and $\alpha\beta\delta$ receptors and (ii) $\alpha\beta\gamma\delta$ receptors (n = 3 per receptor).**

combinations of subunits were characterized: $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2L}$, $\alpha_1\beta_1\delta$, and $\alpha_1\beta_1\gamma_{2L}\delta$ (referred to subsequently as $\alpha\beta$, $\alpha\beta\gamma$, $\alpha\beta\delta$, and $\alpha\beta\gamma\delta$, respectively). Injection of a combination of subunit cDNAs does not necessarily result in all of them assembling into a unique functional receptor. Therefore, we examined the physiologic signalling properties and pharmacology of the expressed combinations to confirm their distinct signatures. Current-voltage (I-V) plots were constructed from the GABA-induced currents between −100 and 0 mV for three subunit combinations (*e.g.,* fig. 1A). Reversal potentials were approximately −23 mV for each, which is consistent with the equilibrium potential for a chloride current in oocytes. As shown in fig. 1B, $\alpha\beta\gamma$ (part i), $\alpha\beta\gamma\delta$ (part i; n = 3 per receptor), and $\alpha\beta\delta$ (part ii; n = 4) all displayed outward rectification at the more positive holding potentials.

Results reported subsequently were obtained from an additional 156 oocytes (from many donors) over the 2-5 days after injection. The four receptor combinations differed in their rate of expression to recordable levels in response to a saturating concentration of GABA (3 mM) and also in their peak currents to maximal GABA (data not shown). $\alpha\beta$ And $\alpha\beta\delta$ isoforms required 3-4 days when peak responses averaged 140.5 ± 59 μS per oocyte (n = 7) and 7.7 ± 3 μS (n = 9), respectively, at −40 mV; in contrast, $\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$ combinations expressed to measurable levels in 1-2 days and yielded peak conductances of 166.1 ± 42.5 μS (n = 9) and 171.95 ± 45 μS (n = 13), respectively, at this time. The apparent threshold for an inward current at −40 mV was between 0.9 μM and 1.8 μM of GABA for all combinations. The receptor subtypes $\alpha\beta$, $\alpha\beta\gamma$, and $\alpha\beta\gamma\delta$ displayed similar dose-response curves, with saturation at or less than 3,000 μM of GABA. Mean Hill slopes were 2.0 ± 0.3, 1.6 ± 0.1, and 1.8 ± 0.1, respectively, indicating cooperativity of GABA binding. The $\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$ receptor subtypes displayed indistinguishable affinity for GABA, with mean EC_{50} values 43.85 ± 1.15 μM and 31.26 ± 1.23 μM, respectively (n = 9 or 10, $P > 0.05$ using analysis of variance); however, they differed significantly from the EC_{50} value of $\alpha\beta$, which was 5.03 ± 1.32 μM (n = 6, $P < 0.05$). $\alpha\beta\delta$ Receptors displayed a less steep dose-response curve (n = 6), saturating at or at less than 200 μM of GABA, with a mean EC_{50} value of 14.13 ± 1.19 μM ($P < 0.05$ *vs.* all combinations) and a Hill slope of 1.2 ± 0.2. This Hill slope was significantly different from the Hill slopes of $\alpha\beta$ and $\alpha\beta\gamma\delta$ combinations ($P < 0.05$) but not from that of $\alpha\beta\gamma$ ($P > 0.05$).

Pharmacologic Strategies to Demonstrate Functional Expression of Receptor Isoforms

One micromole per liter of diazepam markedly enhanced responses from $\alpha\beta\gamma$ (fig. 2A) and $\alpha\beta\gamma\delta$ (fig. 2B) receptors (n = 4 or 5, $P < 0.05$ using *t* test) at subsaturating concentrations of GABA and shifted dose-response curves to the left. Responses of $\alpha\beta$ (not shown) and $\alpha\beta\delta$ (fig. 2C) were not significantly enhanced by 0.333-1.000 μM of diazepam. In contrast to $\alpha\beta\gamma$ receptors, any small modulatory effects of diazepam on $\alpha\beta\delta$ responses ($P > 0.05$) were insensitive to

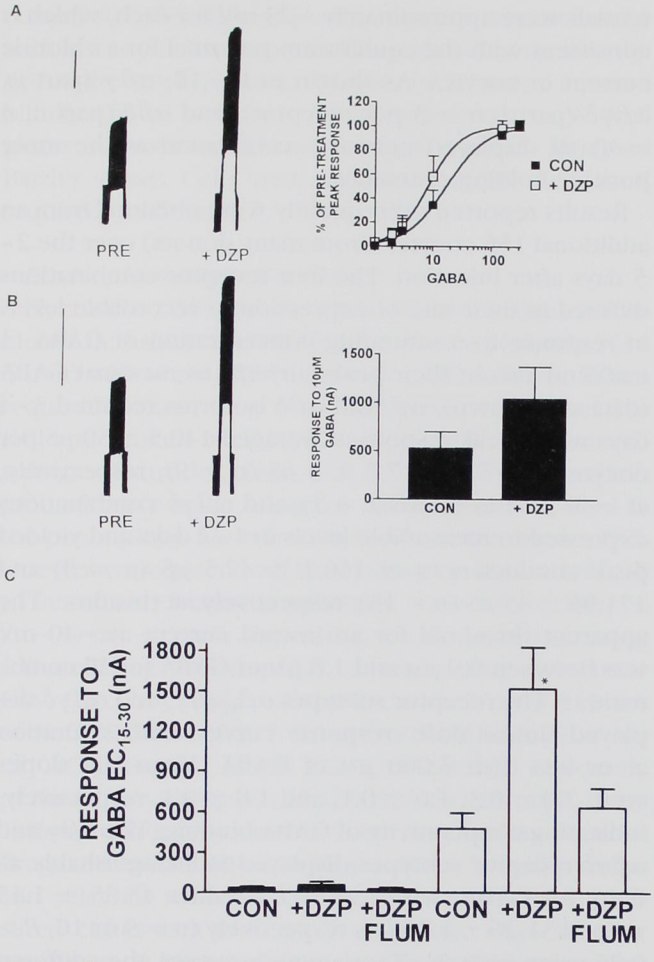

Fig. 2. The modulatory effect of diazepam (DZP) varies with receptor subunit composition. Representative examples of the potentiation by 1 μM of DZP seen at 10 μM of GABA are shown to the left of the compounded results for *(A)* $\alpha\beta\gamma$ receptors—agonist concentration–response curve shifted to the left (n = 4), and *(B)* $\alpha\beta\gamma\delta$ receptors—enhancement of 10 μM of GABA response (n = 5) (scale bars represent 100 nA). *(C)* Bar graph of compounded data showing the effect of 0.333 μM of DZP in the absence and presence of 1 μM of flumazenil (FLUM) for oocytes expressing $\alpha\beta\delta$ (*black bars*) and $\alpha\beta\gamma$ (*white bars*) receptors (mean + SEM; n = 6 and 5, respectively). *Significantly different from relevant control ($P < 0.05$ using one-way analysis of variance).

1 μM of flumazenil (fig. 2C; n = 6). These results confirmed that heteromeric receptors without the γ subunit do not bear a functional benzodiazepine receptor complex.

It has been reported that Zn^{2+} inhibits currents that do not contain a γ subunit and has no significant effect when this subunit is present.[32] In our hands, γ-containing receptors were weakly antagonized by 20 μM of Zn^{2+}; mean reductions were 11% ($P > 0.05$ using t test) and 33% ($P < 0.05$) for $\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$ responses, respectively. Effects on $\alpha\beta$ conductances were unequivocal; responses were reduced by an average of 98% ($P < 0.05$). We also tested $\alpha\beta\delta$ receptors and saw an 87% decrease ($P < 0.05$) in control responses. Representative examples and normalized data are shown in fig. 3A (parts i and ii) and suggest that δ-containing isoforms exhibit intermediate sensitivity to the divalent cation (n = 5 per receptor).

The trivalent ion, lanthanum (La^{3+}), was used to test subunit expression. This has been shown to potentiate $\alpha\beta$ and $\alpha\beta\gamma$[33,34] currents but not those produced by $\alpha\beta\delta$ receptors.[29] We saw a consistent but insignificant increase of GABA-induced conductances in $\alpha\beta$ receptors (140%; $P > 0.05$ using t test) and a significant enhancement of $\alpha\beta\gamma$ receptor responses (240%; $p < 0.05$) in the presence of 600 μM of La^{3+}. When tested on $\alpha\beta\delta$ receptors, 600 μM of La^{3+} marginally depressed responses (70%; $P > 0.05$) and with the $\alpha\beta\gamma\delta$ combination (previously unreported) there were increases (147%; $P < 0.05$; n = 5 or 6 per receptor; fig. 3B). The addition of δ to heterooligomeric receptors appears to reduce sensitivity to potentiation by La^{3+}, albeit not significantly, and the presence of the γ subunit is capable of reversing this effect.

Desensitization

Desensitization was studied in the presence of a saturating concentration of GABA, applied for 50 s, at a holding potential of −40 mV (while monitoring input conductance). As the example in fig. 4A illustrates, for $\alpha\beta$, GABA-induced conductance faded only slightly throughout agonist application (n = 5, $P > 0.05$ using t test). The receptors $\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$ showed a more marked desensitization over the same period, decreasing to 48% and 50% of original levels, respectively (n = 5–7, $P < 0.05$ for each). $\alpha\beta\delta$ Receptors, in common with $\alpha\beta$, were less prone to desensitization, although a significant fading over 50 s was observed ($P < 0.05$). Compounded data are shown in fig. 4B. Overall, these results were interpreted as evidence for the functional expression of δ in the oocyte membrane.

Anesthetic Pharmacology

Before looking at the action of isoflurane on each receptor isoform, we examined the effect of various concentrations of the volatile anesthetic at a fixed GABA concentration (within the range EC_{10-25}) for each particular subunit combination. Graphs were constructed over the range 58–2,879 μM of isoflurane, which produced bell-shaped

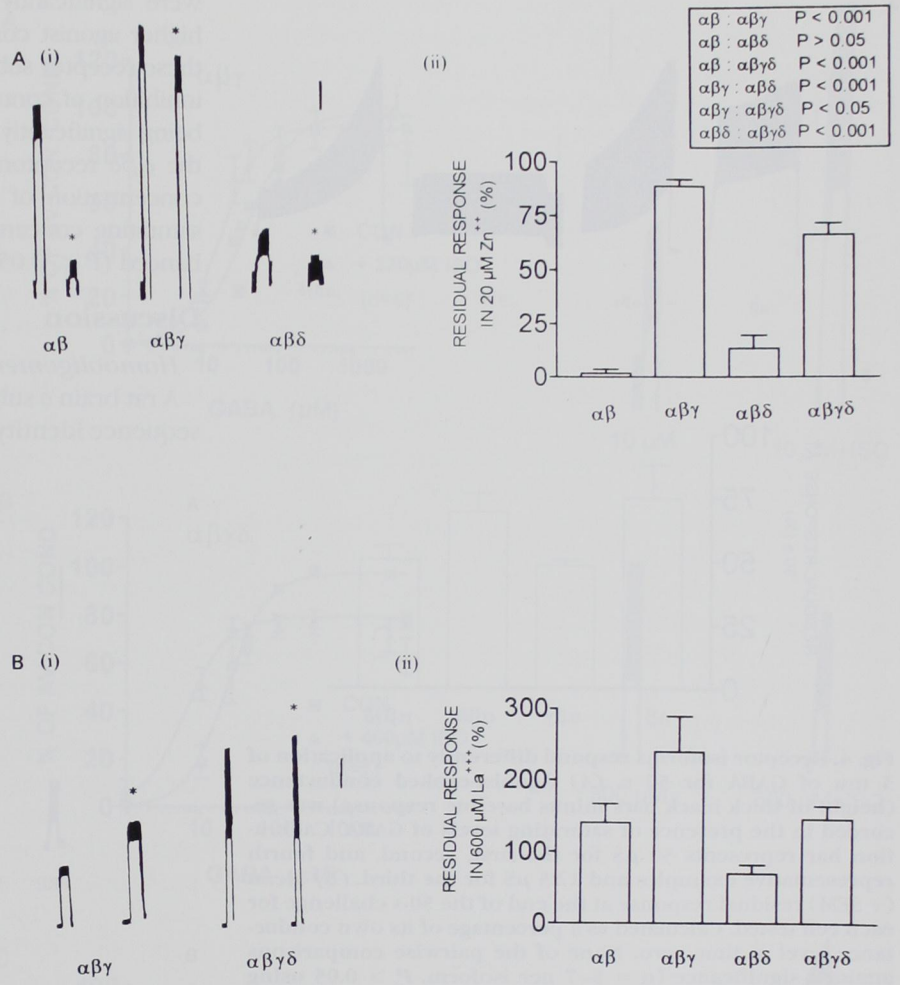

Fig. 3. Modulation of the four receptor subtypes by zinc and lanthanum. *(A)* Zinc. (i) Representative examples showing inhibition of $\alpha\beta$ receptors, little effect on $\alpha\beta\gamma$ responses, and intermediate reduction of $\alpha\beta\delta$ receptors (*presence of 20 μM of zinc; scale bar represents 100, 250, and 100 nA, respectively). (ii) Compounded data for all combinations (mean + SEM; n = 5 per isoform) and a Tukey's all-pairs test (inset; one-way analysis of variance). *(B)* Lanthanum. (i) Representative examples showing potentiation of $\alpha\beta\gamma$ receptors and marginal effect on $\alpha\beta\gamma\delta$ conductance (*presence of 600 μM of lanthanum; scale bar represents 1,000 nA). (ii) Compounded data for all combinations (mean + SEM; n = 5 or 6 per isoform). Only $\alpha\beta\gamma$ and $\alpha\beta\delta$ conductances in the presence of lanthanum were significantly different (all-pairs comparison by one-way analysis of variance).

concentration-response profiles (*e.g.,* fig. 5A) for each of the receptor subtypes (the illustrated curves in fig. 5B depict data up to the maximal only). The isoforms $\alpha\beta$ (n = 2) and $\alpha\beta\gamma$ (n = 7) exhibited similar responses to the anesthetic over this range of concentrations and differed from $\alpha\beta\gamma\delta$ (n = 4); $\alpha\beta$ and $\alpha\beta\gamma$ enhancement saturated at $\approx$440 μM, yielding apparent EC_{50} values of 206 $\pm$ 1 μM and 225 $\pm$ 1 μM, respectively, whereas $\alpha\beta\gamma\delta$ potentiation peaked at $\approx$1,176 μM of isoflurane, with an apparent EC_{50} value of 399 $\pm$ 1 μM. $\alpha\beta\delta$ Receptors (n = 4) demonstrated an intermediate affinity for the modulatory anesthetic; peak enhancement occurred at $\approx$588 μM with an apparent EC_{50} value of 372 $\pm$ 1 μM of isoflurane. The EC_{50} values significantly differed ($P < 0.05$ using one-way analysis of variance); $\alpha\beta$ was not included in this analysis because of limited replication (n = 2). Time-matched treatment blanks (data not shown) resulted in almost superimposeable curves if pre- and posttreatment responses were averaged, indicating that these responses were entirely attributable to the drug, at least for $\alpha\beta\gamma$ (n = 3) or $\alpha\beta\gamma\delta$ (n = 2) receptors. In the two cells expressing $\alpha\beta\gamma\delta$, the blanks indicated a slightly more labile response at the saturating doses of GABA over time, which indicates that we may have marginally underestimated the modulatory efficacy of isoflurane at the higher GABA concentrations on this isoform. Our isoflurane dose-response experiments, however, indicate the $\alpha\beta\delta$ isoform to be modulated profoundly (mean enhancement >10-fold); the rank order of efficacy was $\alpha\beta\delta > \alpha\beta > \alpha\beta\gamma \geq \alpha\beta\gamma\delta$. Isoflurane did not appear to induce any direct agonist effect in the absence of GABA, even at the highest concentration applied.

To probe the modulatory mechanisms further, we then generated concentration-response curves for GABA for

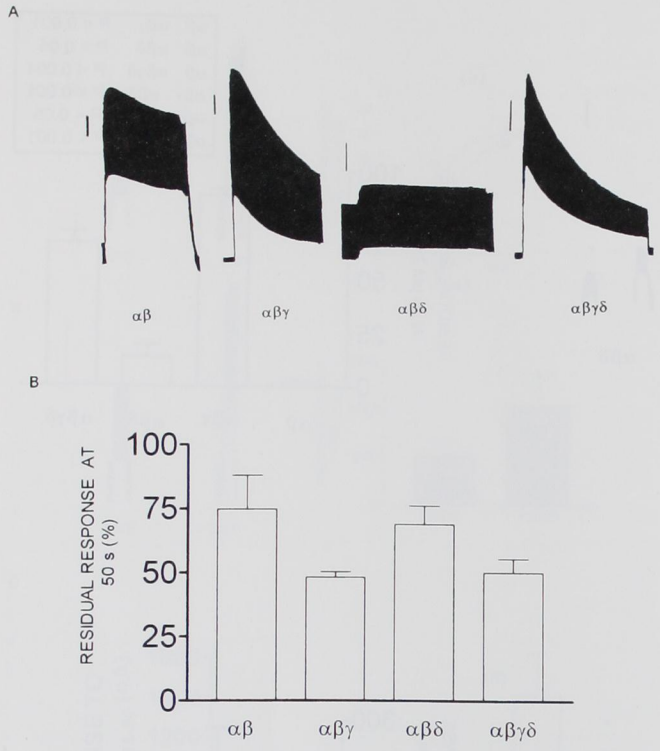

Fig. 4. Receptor isoforms respond differently to application of 3 mM of GABA for 50 s. *(A)* Agonist-evoked conductance (height of thick black bars minus baseline response) was recorded in the presence of saturating levels of GABA. Calibration bar represents 50 μS for the first, second, and fourth representative examples and 12.5 μS for the third. *(B)* Mean (+ SEM) residual response at the end of the 50-s challenge for each cell tested. Calculated as a percentage of its own conductance level at time zero. None of the pairwise comparisons attained significance (n = 5–7 per isoform, $P > 0.05$ using one-way analysis of variance).

three of the subunit combinations before, during, and after exposure to anesthetic at the approximate EC_{50} concentrations indicated previously. Attempts to construct meaningful curves before, during, and after anesthetic using the $\alpha\beta$ receptor were not successful. This was due to the protracted nature of the experiment, marked variation in agonist affinity, and fluctuation in expression levels noted within and between batches of oocytes for this particular receptor. The $\alpha\beta\gamma$ (n = 6) and $\alpha\beta\gamma\delta$ (n = 7) conductances were enhanced as a consequence of increased GABA receptor affinity over the range 1 - 100 μM of GABA (figs. 6A and 6B): There was a parallel leftward shift of this part of the curve, signifying a potentiation of these GABA-activated conductances with no change in Hill slope. Potentiation was most pronounced (up to 600% for $\alpha\beta\gamma$ and 1,100% for $\alpha\beta\gamma\delta$) at low agonist concentrations, and EC_{50} values were significantly altered ($P < 0.0001$ using t test). At higher agonist concentrations (100 - 5,000 μM of GABA), these receptor subtypes displayed no potentiation or even inhibition of control responses, with $\alpha\beta\gamma\delta$ conductances being significantly reduced ($P < 0.05$) at saturation. When the $\alpha\beta\delta$ receptors were tested with their apparent EC_{50} concentration of isoflurane, the maximum responses to saturating concentrations of GABA were significantly enhanced ($P < 0.05$; fig. 6C).

Discussion

Homooligomeric δ Receptors

A rat brain δ subunit, which shares approximately 35% sequence identity with cloned α and β subunits,[25,26] has

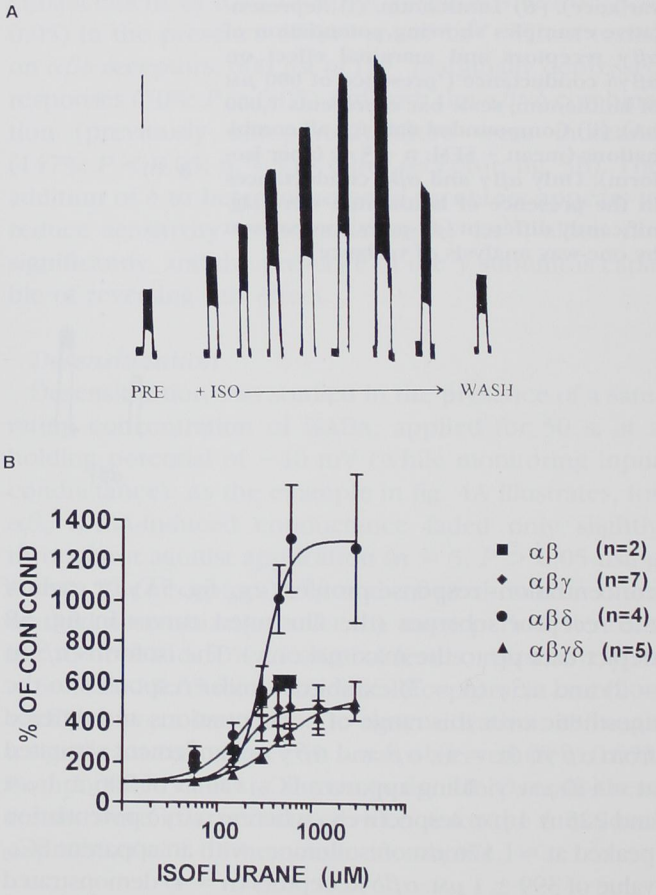

Fig. 5. Concentration dependency of isoflurane modulation for the four receptor isoforms. *(A)* Representative example ($\alpha\beta\gamma\delta$) for the nominal concentrations (left to right) of 58, 147, 294, 440, 587, 1,175, and 2,879 μM of isoflurane (ISO) on response to 5 μM of GABA, depicting the bell-shaped nature of the effect seen for all isoforms (scale bar represents 1,000 nA). *(B)* Compounded data (mean ± SEM) for each combination (fitted by nonlinear regression up to the maximum only for each curve).

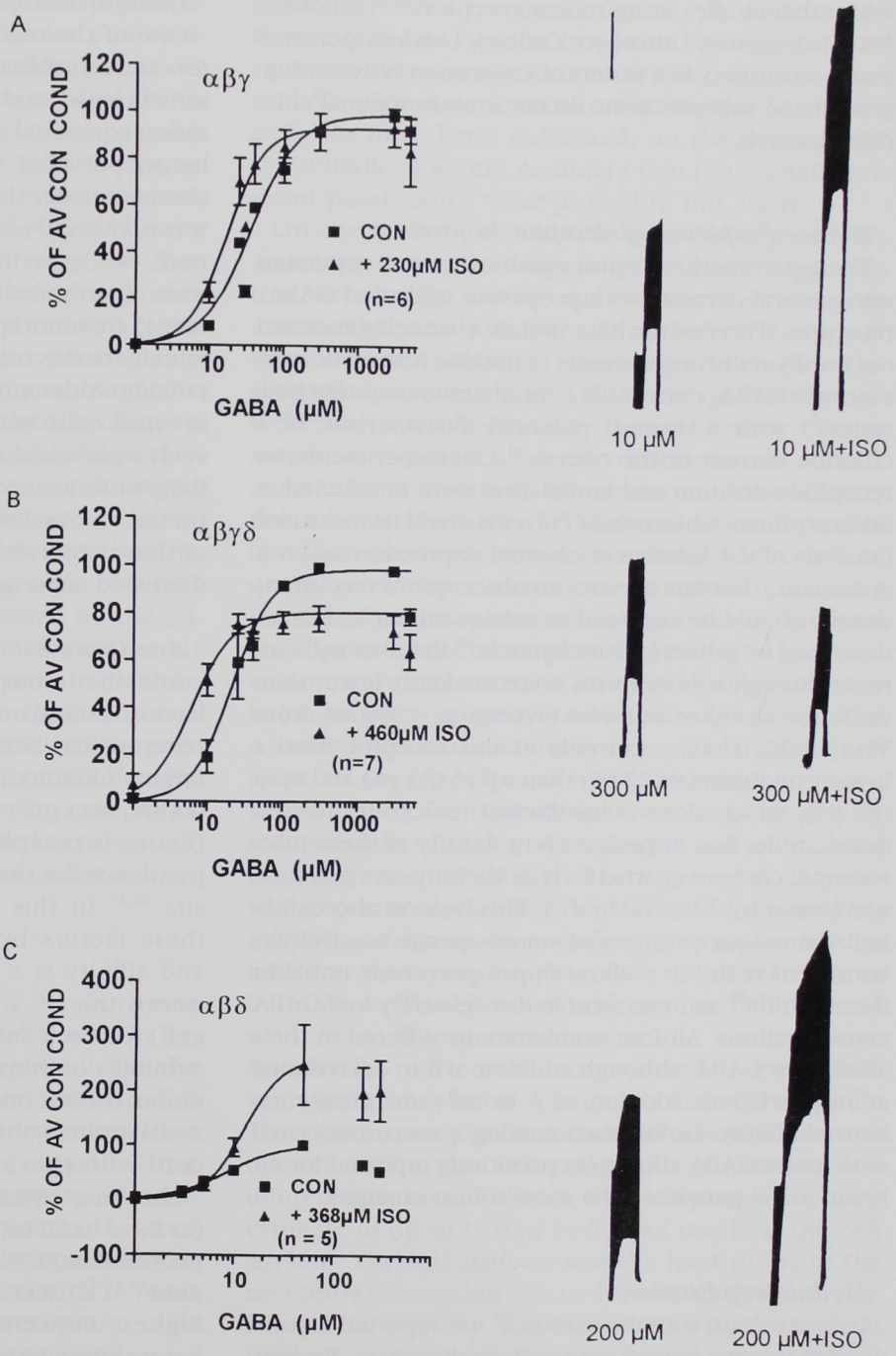

Fig. 6. Compounded data (mean ± SEM) and representative examples at a single dose (right column), showing the response of various receptor isoforms in the absence (*solid sqares*) and presence (*open triangles*) of isoflurane. *(A)* $\alpha\beta\gamma$ Receptors—EC_{50} significantly reduced from 49.9 ± 1.2 to 20.8 ± 1.2 μM by the anesthetic (n = 6, $P < 0.0001$ using *t* test) with a marginal depression of maximal response ($P > 0.05$) at saturating GABA concentrations. *(B)* $\alpha\beta\gamma\delta$ Receptors—EC_{50} significantly reduced from 25.0 ± 1.2 to 6.3 ± 1.4 μM (n = 7, $P < 0.0001$) and significant depression of the maximal response ($P < 0.05$). *(C)* $\alpha\beta\delta$ Receptors—Isoflurane evoked a marked enhancement of responses even to saturating concentrations of GABA (n = 5, at 200 μM, $P < 0.05$). (Scale bar represents 200, 2,000, and 200 nA, respectively).

been subjected to only limited physiologic and pharmacologic characterization.[29] These rodent subunits were reported to form functional GABA-activated (glycine-insensitive) chloride channels as homooligomers in transfected cell lines,[25] but this may reflect the presence of endogenous GABA subunits in the human embryonic kidney cells used.[35] Electrophysiologic experiments suggest that homooligomeric β_1[36] and ρ receptors (*e.g.*, refs. 21 and 23) can generate functional channel complexes. Our own work with human cDNA, in common

with other studies using rodent receptors[28,29] (SJ Moss, MRC Laboratory, University College, London, personal communication), in a variety of expression systems, suggests that δ subunits alone do not form functional chloride channels.

Passive Properties of Receptor Isoforms

The heteromeric receptor combinations all generated unequivocal currents with properties typical of $GABA_A$ receptors. Three of the heteromeric channels generated outwardly rectifying currents (a notable feature of macroscopic $GABA_A$ currents in central nervous system neurones[37]) with a reversal potential characteristic of a chloride current in the oocyte.[38] Our experiments on receptor activation and modulation were conducted at arbitrary times when robust currents could be measured (analysis of the kinetics of channel expression was not systematic). For this reason, our observations on current density should be regarded as semiquantitative, but, as described by others for rat channels,[29] the maximal currents through $\alpha\beta\delta$ isoforms were markedly lower than those for all other isoforms (averaging <200 nA from Vh-40 mV). Unitary currents in this receptor have a lower conductance (22 pS) than $\alpha\beta\gamma\delta$ (33 pS) and $\alpha\beta\gamma$ (30 pS), which alone is insufficient to account for the drastic reduction in peak current density of these macroscopic currents (particularly as their mean open time was longer by 20- to 80-fold[29]). This isoform also exhibited the unique property of noncooperative activation consistent with the shallow slopes previously noted in the rat study[29] and was saturated at relatively low GABA concentrations. All four combinations differed in their affinity for GABA, although addition of δ to $\alpha\beta$ reduced affinity, whereas addition of δ to $\alpha\beta\gamma$ marginally enhanced affinity. Isoforms containing γ were associated with lower GABA affinity (as previously reported for rat brain[39]) and generated the most robust currents.

Modulatory Profiles

In a previous communication,[40] we reported a paradoxical inverse agonist response to diazepam. By lowering solvent levels and using fixed dimethylsulfoxide concentrations in saline solutions throughout, we found that the $\alpha\beta\delta$ combination is insensitive to benzodiazepine modulation. The sensitivity to zinc and lanthanum, together with effects on maximal current density, affinity, and Hill slope, strongly suggests that δ subunits are functionally co-expressed in oocytes.

Desensitization

One of the unique signatures of δ-containing receptors in recombinant rat receptors was their relative insensitivity to, and rapid recovery from, acute desensitization compared with $\alpha\beta\gamma$.[29] In our hands, using much longer pulses of saturating GABA and measuring conductance fade, the extent of desensitization observed was $\alpha\beta\gamma \approx \alpha\beta\gamma\delta$ ($\approx$50%) $> \alpha\beta\delta \approx \alpha\beta$. On a cautionary note, our experiments in the oocyte suggest that currents (particularly those large responses in the μA range) are more labile than conductance measurements, which, for this reason, we used to produce the previous ranking. Although limiting or dissipated ionic gradients in small cells under whole cell clamp are less likely, such a phenomenon might explain the relative insensitivity of tiny currents to fading (almost invariably interpreted as desensitization).[29] Some of the implications of these observations for anesthetic pharmacology are discussed subsequently.

Anesthetic Subunit Dependence

Anesthetic interactions with ionotropic receptors have attracted much recent attention, and the $GABA_A$ receptor has been proposed as a unifying molecular target.[13] Estimation of the amount of potentiation of $GABA_A$ receptor isoforms and their affinity for anesthetics is complicated considerably by a strong dependence on the extent of occupation of the GABA site.[22,24] In this study, we endeavored to equalize these factors by studying the modulatory efficacy and affinity at a concentration of GABA strictly between the EC_{10} and EC_{25} levels (measured in each cell) for each subunit combination. Responses in all subunit combinations were enhanced by isoflurane under these conditions, and no analogies with benzodiazepine subunit dependence can be seen (in accord with previous studies[24]).

No direct agonist effects in the absence of GABA (as have been reported for barbiturates, steroids, and propofol and which are markedly subunit dependent[11,16,41]) were evoked by isoflurane, even at the highest concentrations. Volatile anesthetics have been shown to activate chloride currents directly in expression systems[42] and primary cultures.[43] These divergent results may reflect subunit dependence, the presence of trace amounts of experimental GABA, or different rates of anesthetic administration. The δ subunit does quantitatively regulate anesthetic affinity and efficacy: Both $\alpha\beta\delta$ and $\alpha\beta\gamma\delta$ have a lower affinity for isoflurane than the other combi-

nations, but the peak modulatory response is greater by approximately twofold for the former combination. Isoflurane, at or near its EC_{50} value, enhances both $\alpha\beta\gamma\delta$ and $\alpha\beta\gamma$ currents by increasing GABA receptor affinity, but maximal responses are to some extent depressed. In this respect, $\alpha\beta\delta$ receptors were again exceptional, as even maximal responses at or near saturating concentrations of GABA were enhanced by isoflurane. This result suggests that isoflurane may be increasing the $\alpha\beta\delta$ receptor probability of opening (the slope of the modulated curve was not significantly different from the control, suggesting that cooperative activation was not facilitated by anesthetic) or that the anesthetic is increasing the number of $\alpha\beta\delta$ receptors available for activation. One mechanism for the latter proposal might be through repriming of desensitized receptors or interference with the onset of fast desensitization. The response time in oocytes (large cells, $\approx$1 mm in diameter) is undoubtedly constrained by the superfusion method used in this study. Elegant recent work on recombinant nicotinic receptors in cell-free patches[10] demonstrates a fast phase of desensitization that would almost certainly be masked by concurrent activation in our study. The submolecular mechanisms for the low current-carrying capacity of this subunit combination (or its limited expression), together with its unique response to isoflurane, remain unresolved.

Anesthetics in the Brain

The number of theoretical subunit combinations within a pentameric $GABA_A$ receptor[3] is well in excess of 10,000, but it has been suggested that the number of functional isoforms is more likely to fall between 17 and 850.[2] Most $GABA_A$ receptors, at least in rat brain, are composed of $\alpha_1\beta_2\gamma_2$ subunits.[4] For this and other combinations, stoichiometry is uncertain, but one article has suggested that 2α, 1β, and 2γ may be favored.[44] Despite initial suggestions to the contrary, expression of $\alpha\beta$ alone results in functional benzodiazepine-insensitive receptors, and their existence in restricted brain regions cannot be excluded (*e.g.,* ref. 2). Some groups propose that, in the brain, γ and δ subunits are largely mutually exclusive,[4] although one report suggests that γ_2 and δ co-assemble to produce a receptor with novel pharmacology.[27] In a large number of regions, RNA for δ colocalizes with those for α_1, α_4, and β_2 (principally the thalamic nuclei)[26]; however, mapping of subunit gene expression[45] suggests that α_1, β_1, and γ_2 may form a receptor isoform in certain specific areas of rat brain (δ is also distributed in some of these locations[26]). Although no localization studies on human isoforms have been published, on the basis of the these findings we are confident that functionally relevant possibilities were probed in this study.

On the basis of somatosensory-evoked potentials and single-unit recordings in rats, Angel[46] suggested that structurally diverse anesthetics (including the commonly used inhalational agents) first impede transmission of sensory information at the level of the thalamic relay nuclei with further depressant effects on cortical circuits. Ligand-binding experiments also suggest anatomic differences in volatile anesthetic modulation, which may reflect differential subunit sensitivity.[47,48] Subunit dependence of anesthetic action is now acknowledged.[5,24] Our own quantitative experiments extend this database and provide further evidence for selective interaction with proteinaceous targets. Regarding whether δ subunits are important in conferring such differential sensitivity *in vivo,* we can only speculate. The $\alpha\beta\gamma\delta$ isoform had a modulatory profile largely equivalent to the widespread $\alpha\beta\gamma$[4]; although it had a lower affinity for isoflurane, it was associated with a depression of maximal responses and an increase in GABA receptor affinity. The depression of maximal response may underpin paradoxical reports that inhalational agents do not affect or even depress inhibitory postsynaptic potentials (*e.g.,* ref. 49) in brain slices. The effects of isoflurane in the this study were seen at concentrations in the clinical range (minimum alveolar concentration-equivalent isoflurane has been cited as 320[7]-510 μM[20]). Throughout, we cite nominal calculated concentrations (based on gas chromatography of saturated and diluted aqueous solutions), which represent an overestimate of up to 12% of isoflurane reaching the cell surface. The $\alpha\beta\delta$ isoform was the least prone of the receptors (excepting $\alpha\beta$) to desensitization and, perhaps coincidentally, was associated with the highest intrinsic modulatory efficacy, albeit at relatively high concentrations. The presence of supersensitive/hyperresponsive anesthetic receptors in the brain with a discrete anatomic location is an attractive hypothetical concept. On the basis of the relatively low affinity for isoflurane and their inherently low current-carrying capacity, we do not believe that $GABA_A$ receptors consisting of $\alpha\beta\delta$ subunits alone represent

such a target. Magnetic resonance image-based scanning and the increasing availability of knockout mice for ion channel subunits may contribute to this debate in the foreseeable future.

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