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Isobolographic Analysis of the Interactions between Midazolam and Propofol at $GABA_A$ Receptors in Embryonic Mouse Neurons

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Background: **Clinical studies suggest that midazolam and propofol interact synergistically to induce hypnosis, but these drugs do not interact synergistically to prevent movement in response to noxious stimuli. The mechanisms underlying these interactions are not certain but may occur at the level of the γ-aminobutyric acid A ($GABA_A$) receptor.**

Methods: **The authors evaluated the interactions between propofol and midazolam in modulating $GABA_A$ receptor activity in embryonic hippocampal neurons. The effects of midazolam and propofol on peak current evoked by submaximal concentrations of GABA were studied using the patch clamp method. Isobolographic analysis was undertaken by constructing concentration–response curves for midazolam and propofol alone and then evaluating the potency of combinations of midazolam and propofol. In other experiments, the concentration of GABA was increased and flurazepam was substituted for midazolam.**

Results: **Isobolographic analysis confirmed that midazolam and propofol interact synergistically to enhance currents evoked by low concentrations of GABA (1 μM). However, when the concentration of GABA was increased to 3 μM, the interaction was additive. The interaction between flurazepam and propofol was also additive for enhancement of currents evoked by 3 μM GABA.**

Conclusions: **The interaction between midazolam and propofol was critically dependent on the concentration of GABA: Synergism was evident at low concentrations of GABA, but an additive interaction was apparent when the concentration of GABA was increased. Changes in $GABA_A$ receptor function may underlie the synergistic interaction between propofol and midazolam for clinical effects such as hypnosis. The clinical implication of the results is that the benefits of synergism observed at one concentration ratio of these drugs may not be apparent at another. (Key words: Anesthesia; ion channels; synergism.)**

GENERAL anesthesia is a spectrum of clinical effects that include hypnosis, amnesia, analgesia, and immobility in response to surgical stimuli. Anesthesia usually is achieved by administering a combination of drugs with different pharmacologic properties. Midazolam and propofol often are used, in combination, to induce the transition to unconsciousness. This practice is based on animal and human studies that indicate that propofol and midazolam interact synergistically for hypnosis.[1,2] Synergism occurs between a pair of drugs when the potency of the drug combination is greater than the potency expected from the additive effect of each drug alone.[3] Although midazolam and propofol interact synergistically for hypnosis, at higher concentrations these drugs do not interact synergistically to prevent movement in response to noxious stimuli.[1,2] The observation that synergism occurs for some but not all of the effects of general anesthetics agrees with the concept that the different behavioral components of the anesthetic state are mediated by different underlying cellular mechanisms.[4–6]

A current focus in anesthesiology is identifying which receptors mediate the different behavioral aspects of anesthetic actions. Propofol and midazolam (similar to many other intravenous anesthetics) are thought to exert their neurodepressive properties in part by enhancing γ-aminobutyric acid (GABA)-mediated neurotransmission.[7–13] (For a review of this, see Carlson *et al.*[12]) GABA, the major inhibitory transmitter in the central nervous system, activates several subtypes of receptors.[13–15] The $GABA_A$ receptor mediates fast inhibitory synaptic neurotransmission and is found in nearly every neuron in the brain.[14,16] It is a pentameric heterooligomer that con-

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tains an integral chloride-permeable ion channel and is composed of multiple subunits that have been classified in several structural categories (α1–6, β1–3, γ1–3, δ, and ϵ).[14,17,18] $GABA_A$ receptors contain a multiplicity of allosteric binding sites for neurodepressive drugs, including benzodiazepines, propofol, barbiturates, and anesthetic steroids.[13,14,19,20] These compounds modify receptor function and increase the probability of opening of the associated chloride channel. In most neurons, the resulting increase in chloride conductance causes membrane hyperpolarization and the shunting of excitatory input. Thus, enhancement of $GABA_A$ receptor activity reduces the likelihood of generating an action potential in the postsynaptic neuron.

Midazolam is a relatively short-acting benzodiazepine used clinically for its sedative, hypnotic, and anticonvulsant properties. Midazolam potentiates $GABA_A$ receptor function primarily by increasing the binding affinity of the receptor for GABA (for a review see Johnston[13]). Electrophysiologic studies have shown that classic benzodiazepines, such as diazepam, reversibly potentiate $GABA_A$ receptor-mediated currents and increase the frequency of channel opening.[19,21–23] Furthermore, hypnotic benzodiazepines, such as midazolam, flurazepam, and diazepam, prolong the time course of inhibitory postsynaptic currents and increase their amplitude in large inhibitory synapses.[23] The effects of midazolam on $GABA_A$ receptor function are blocked by the benzodiazepine antagonist flumazenil (Ro 15-1788; Hoffmann-LaRoche, Ltd., Mississauga, Ontario, Canada). Flumazenil also reverses the behavioral effects of midazolam, suggesting that binding to a specific site on the postsynaptic $GABA_A$ receptor mediates the clinical effects of midazolam.[24–26]

The intravenous general anesthetic propofol also binds to a site on the $GABA_A$ receptor. However, flumazenil fails to influence either the actions of propofol on the $GABA_A$ receptor or the clinical properties of propofol.[11,26–28] Propofol increases the binding affinity of the receptor for GABA, increases the frequency of channel opening, and slows the decay of inhibitory postsynaptic currents.[29,30] However, dissimilar to benzodiazepines, propofol (and other intravenous anesthetics, including alphaxalone, certain neuroactive steroids, etomidate, and pentobarbital) directly activates the $GABA_A$ receptor in the absence of GABA and influences the kinetics of receptor desensitization.[9–11,29]

The purpose of this study was to investigate the interactions of midazolam and propofol for activation of $GABA_A$ receptors. We used the patch-clamp method to evaluate current evoked by subsaturating concentrations of GABA recorded from cultured hippocampal neurons. Because the effects of anesthetics on $GABA_A$ receptor function are strongly influenced by the agonist concentration, several concentrations of GABA were studied.[20] Furthermore, because synergism depends on the relative concentrations of the two drugs (or the concentration ratio),[3] we evaluated several concentrations of propofol.

Methods

Cell Cultures

Animal treatment protocols were approved by the Animal Care Committee of the University of Toronto. Cultures of embryonic hippocampal neurons were prepared from Swiss white mice, as previously described.[29] Briefly, fetal pups (17 days *in utero*) were removed from mice killed by cervical dislocation. The hippocampi were dissected from each fetus and placed in an ice-cooled culture dish. Neurons were then dissociated by mechanical trituration using two Pasteur pipettes (tip diameter, 150–200 μm) and plated on 35-mm culture dishes at a density of approximately 1×10^6 cells/ml. The culture dishes were coated with collagen (from calf skin) or poly-D-lysine (Sigma Chemical Company, St. Louis, MO). For the first 10 days *in vitro*, cells were maintained in minimal essential media supplemented with 10% fetal bovine serum and 10% horse serum (Life Technologies, Grand Island, NY). The neurons were cultured at 36.5°C in a 5% carbon dioxide–95% air environment. After the background cells had grown to confluence, 0.1 ml of a mixture of 4 mg 5-fluorodeoxyuridine and 10 mg uridine in 20 ml minimal essential media was added to the extracellular solution to reduce the number of dividing cells. Subsequently, the media was supplemented with 10% horse serum and changed every 3 or 4 days. Cells were studied 12 to 16 days after dissociation. For all reported results, data were acquired from the cells from at least three different dissections.

Whole-Cell Recordings

Before recording, neurons were rinsed with an extracellular recording solution containing 140 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 25 mM N-2-hydroxy-ethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 33 mM glucose, 1 mM $MgCl_2$, and 0.3 mM tetrodotoxin (TTX) (*p*H 7.4, 325–335 mOsm). Whole-cell patch-pipettes (3–10 MΩ) were fabricated from borosilicate glass capillary tubing and filled with a solution containing 140 mM choline-Cl,

10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 10 mM tetraethylammonium chloride (TEA-Cl), 2 mM $MgCl_2$, and 4 mM Mg-ATP (*p*H 7.4, 290–300 mOsm). Neurons were voltage-clamped at −60 mV, and all experiments were conducted at room temperature. Whole-cell currents were recorded using the Axopatch 1-D amplifier (Axon Instruments, Foster City, CA). Data were digitized, filtered (2 kHz), and acquired on-line using the pClamp 6 program (Axon Instruments). A multibarreled perfusion system was used to achieve a rapid exchange of solutions. Solutions flowed continuously through the barrels, and the barrel position was controlled by a computer-driven DC stepping motor (Vexta; Oriental Motor USA, Torrance, CA). The amplitude of GABA-evoked whole-cell currents gradually, and irreversibly, decreases progressively to a stable amplitude. Presumably, this slow "run-down" of current results from changes in the intracellular contents of the cell after disruption of the membrane patch. To allow for run-down, no responses were used for data analyses until 10 min after breakthrough.

Intralipid, at concentrations equivalent to those used in the experiments, had no effect on the amplitude or time course of GABA-activated current. However, propofol at high concentrations (approximately 1 to 6 μM) directly activated the $GABA_A$ receptor in hippocampal neurons.[11,29] In our experiments, it was not possible to distinguish current resulting from receptor activation by propofol from the GABA-evoked (but propofol-modulated) response. Propofol-activated current could not simply be subtracted from the total current because GABA enhances propofol-induced receptor activation. Therefore, for this analysis, we did not try to differentiate between propofol- and GABA-evoked currents.

An underlying assumption of the isobolographic analysis is that the peak current accurately reflects receptor activation. However, the peak current could be underestimated because of desensitization that occurs during the onset of the response. Experiments were designed to minimize the effect of desensitization on the amplitude of the peak response. To ensure that the rate of activation was considerably faster that the rate of desensitization, low concentrations of GABA (less than the mean effective concentration [EC_{50}]) were used to elicit control responses. This analysis also assumed that the peak current was measured during equilibrium conditions. However, the rate of equilibration of propofol with the $GABA_A$ receptor is likely to be slower than that of GABA or midazolam.[29] Nevertheless, the effect of slow equilibration of propofol was minimal because the current gradually increased to a relatively stable amplitude.

Data Analysis

Whole-cell currents were analyzed using the CLAMPFIT program of the pClamp software (Axon Instruments), and the concentration–response analysis was performed using Graph Pad Prism software (Graph Pad, San Diego, CA). Current amplitudes were plotted against the drug concentrations and fit using a standard logistic equation

$$I = I_{max}(1 + EC_{50}/C)^n)$$

where I is the current amplitude, I_{max} is the maximum current amplitude, C is the concentration of the agonist, EC_{50} is the concentration of agonist that produced 50% of the maximal response, and n is the Hill coefficient. Because the ability of anesthetics to enhance GABA-evoked currents is greatest at low concentrations of agonist, control responses were elicited by low concentrations of GABA (0.3, 1, and 3 μM). These concentrations of GABA were selected based on our previous studies[29] of GABA-evoked currents from embryonic hippocampal neurons. In the current study, 0.3, 1, and 3 μM GABA produced 3%, 9%, and 23%, respectively, of the peak current evoked by a saturating concentration of GABA (600 μM). Thus, the concentrations of GABA used for control responses were much lower than the EC_{50} value.

Analysis of Drug Interactions. The potency of a drug is measured as a dose (or concentration) that produces a specified effect level. In this study, two methods were used to determine whether midazolam and propofol interacted in an additive, supraadditive (synergistic), or subadditive (antagonistic) manner.[31–34] In the first series of experiments, a fixed-concentration model was used to evaluate drug interactions among a range of drug and agonist concentrations.[3] The fixed-concentration model predicts that the combination of drug 1 and drug 2 will produce an effect equal to the predicted sum of each effect alone (or the theoretical additive response). This analysis assumes that the concentrations of the drugs used for the analysis fall on the linear portion of the dose–response curve.[32] Furthermore, although the drugs may differ in potency, it also assumes that the shapes of the dose–response curves are similar. The concentration–response curves for midazolam- and propofol-induced enhancement of $GABA_A$ receptor activity may not be parallel, because drugs that modulate the $GABA_A$ receptor can greatly differ in potency. Thus, the potency ratio of these two drugs could change with the level of effect, and synergism may not be detected unless a wide range of concentrations is tested.

In the second series of experiments, drug interactions were evaluated using isobolographic analysis. The isobolographic analysis evaluates a range of drug concentrations and considers equieffective concentrations of two drugs and the combination of the two drugs that yield a specified level of effect. The isobologram is a Cartesian plot of pairs of doses that, in combination, produce a specified level of effect. The isobologram was constructed by determining the potency of propofol and midazolam alone to enhance GABA-evoked currents. We arbitrarily selected an effect level of 300%, or three times the amplitude of current evoked by a low concentration of GABA alone (*i.e.*, a 200% increase in the response to GABA alone). The concentration of GABA was chosen such that even a response that was three times the control response was not near saturation. The concentrations of midazolam and propofol that increased the GABA-evoked responses by a factor of three were then plotted on the x and y axes of the isobologram, respectively. A diagonal line, called the "isobol of additivity," was then joined to these two end points. Subsequently, we selected a lower, fixed concentration of midazolam and determined the concentration of propofol that produced (in combination with midazolam) the desired effect level. If the two drugs have an additive interaction, the concentration of propofol would lie on the isobol of additivity.[30,32] However, if the two drugs produce a synergistic interaction, the concentration of propofol would fall below the isobol of additivity because less propofol would be needed to produce the desired effect. Conversely, if the drugs interact in an antagonistic or subadditive manner, the propofol dose would lie above the isobol of additivity. The advantage of this method is that it allows for a rigorous statistical test of synergy. Statistical considerations for isobolographic analysis have been described before.[32–34]

Drugs and Other Chemicals

Propofol was prepared from Diprivan (Zeneca Pharma, Missassauga, Ontario, Canada), where each milliliter of Diprivan contained 10 mg 2,6 di-isopropylphenol, 100 mg soybean oil, 12 mg egg lecithin, and 22.5 mg glycerol. Stocks of propofol (1 and 10 mM) were prepared using filtered water every 1 to 3 days and were stored at 4°C. Midazolam was prepared from Versed (Hoffmann-LaRoche, Missassauga, Ontario, Canada) and dissolved in extracellular solution. Each milliliter of Versed contained 1 mg midazolam, 0.1 mg disodium edetate, 10.45 mg benzyl alcohol, and 8 mg NaCl. In addition, purified midazolam (donated by Hoffmann-LaRoche) was dissolved in dimethylsulfoxide. There was no difference in the potentiation of GABA-evoked currents when midazolam was prepared from Versed *versus* the purified compound. Flurazepam was also dissolved in dimethylsulfoxide. Other chemicals, including EGTA, HEPES, and potassium chloride, were purchased from Fluka Biochemika (Bucks, Switzerland); dimethylsulfoxide was received from Fischer Scientific (Fairlawn, NJ), CNQX was received from Tocris Cookson (St. Louis, MO), TTX was received from Precision Biochemicals, (Vancouver, British Columbia, Canada), and NaCl, $CaCl_2$, glucose, and NaOH were received from BDH (Toronto, Ontario, Canada). Unless otherwise specified, all other compounds were purchased from Sigma Chemical Company.

Statistical Analysis

Data are presented as the mean ± SEM or are normalized to control values, unless otherwise indicated. An analysis of variance was used to compare multiple groups of data. The EC_{50} values and Hill slopes of different concentration–response curves were compared using paired Student's *t* tests. Statistical significance was accepted when *P* was less than 0.05.

Results

Fixed Concentration Ratio and the Effects of Propofol, Midazolam, and Flurazepam in Hippocampal Neurons

The application of exogenous GABA (0.1–600 μM) elicited inward current in all hippocampal neurons that were voltage-clamped at −60 mV. Midazolam (0.5 μM) and propofol (10 μM) both increased the amplitude of currents evoked by subsaturating concentrations of GABA (3 μM), as illustrated in figure 1A. The peak amplitude of the current activated by 3 μM GABA, in the absence or presence of midazolam, propofol, or the combination of midazolam and propofol, was less than the maximal current evoked by a saturating concentration of GABA (600 μM). GABA (3 μM)-activated currents were enhanced by midazolam and propofol by factors of 1.45 ± 0.21 and 3.44 ± 0.41, respectively, whereas the combination of midazolam and propofol enhanced currents by a factor of 4.15 ± 0.54 (n = 8; fig. 1B). The increase in the peak current, recorded in the presence of propofol and midazolam, was not significantly different from the predicted additive response ($P > 0.05$; Student's *t* test).

Similar results were obtained when 0.5 μM flurazepam

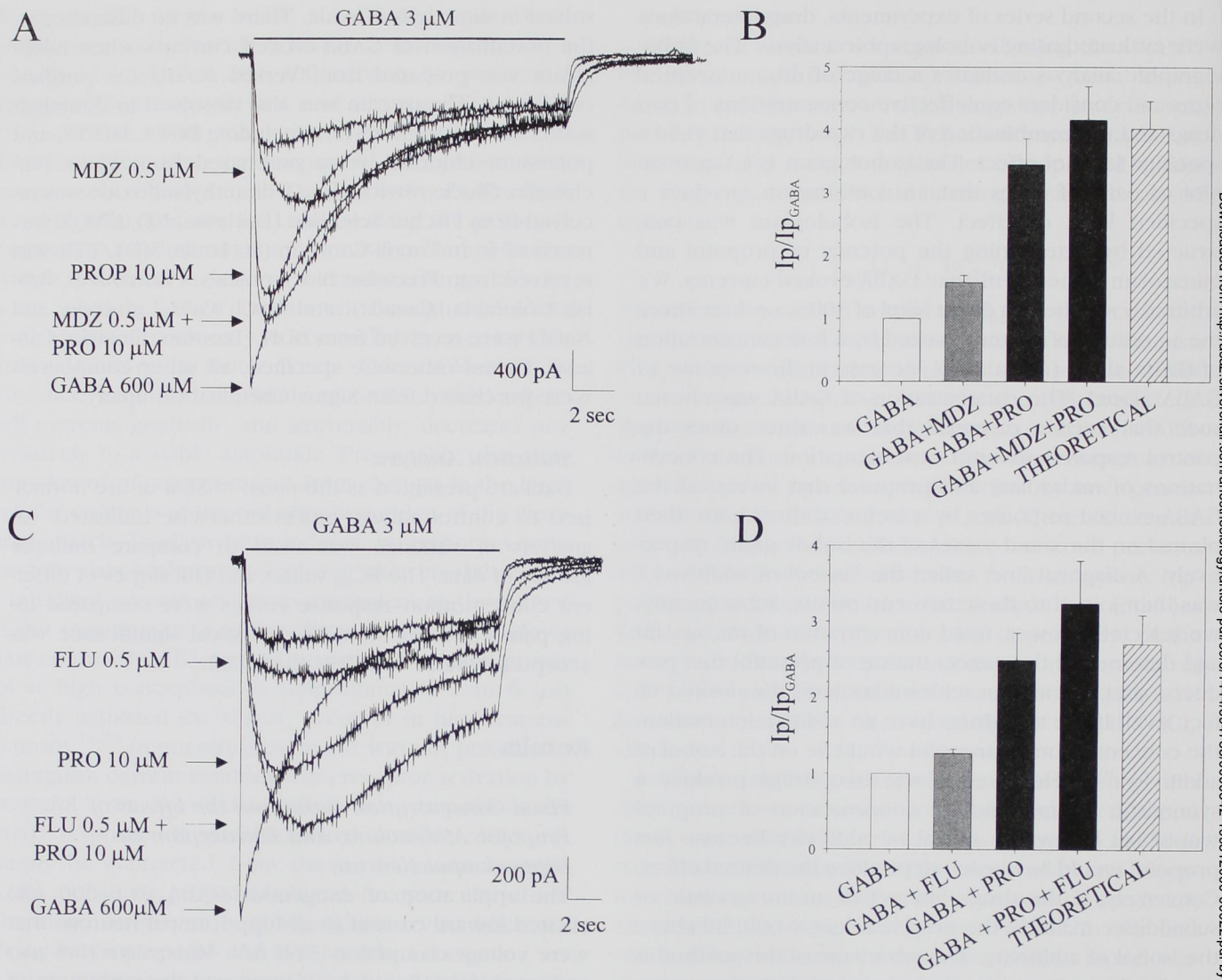

Fig. 1. Additive interactions between propofol and midazolam, or propofol and flurazepam, in hippocampal neurons. (*A*) Enhancement by 10 μM propofol, 0.5 μM midazolam, or the combination of midazolam and propofol of current activated by 3 μM γ-aminobutyric acid (GABA) in hippocampal neurons. Responses shown are superimposed. The peak amplitude of current evoked by 3 μM GABA, in the absence of presence of drug, was less than the current evoked by 600 μM GABA. Responses were reversibly inhibited by bicuculline and reversed polarity at membrane potentials close to the chloride equilibrium potential (not shown). (*B*) The bar graphs indicate peak currents normalized to the amplitude of current evoked by 3 μM GABA. (*C,D*) The interaction between midazolam and propofol, or flurazepam, and propofol was additive, because the measured response was not significantly different from the theoretical additive response ($P < 0.05$, Student's *t* test).

was substituted for midazolam; 0.5 μM flurazepam, 10 μM propofol, and 10 μM propofol and 0.5 μM flurazepam increased the peak current by a factor of 1.26 ± 0.06, 2.49 ± 0.36, and 3.24 ± 0.56, respectively (n = 7; fig. 1C). These data suggest that midazolam and propofol or flurazepam and propofol had an additive interaction with respect to their ability to enhance the peak current evoked by 3 μM GABA in hippocampal neurons (fig. 1D).

The ability of anesthetics to enhance GABA-evoked responses is influenced by both the concentration of the anesthetic and the concentration of GABA.[20] In the next series of experiments, the concentration of propofol was decreased from 10 to 1 μM, whereas the concentration of midazolam was unchanged. Midazolam at doses of 0.5 μM, 1 μM propofol, and 1 μM propofol plus 0.5 midazolam enhanced currents evoked by 3 μM GABA by a factor of 1.62 ± 0.40, 1.89 ± 0.60, and 2.72 ± 1.08 (n = 5), respectively. The interaction between midazolam and

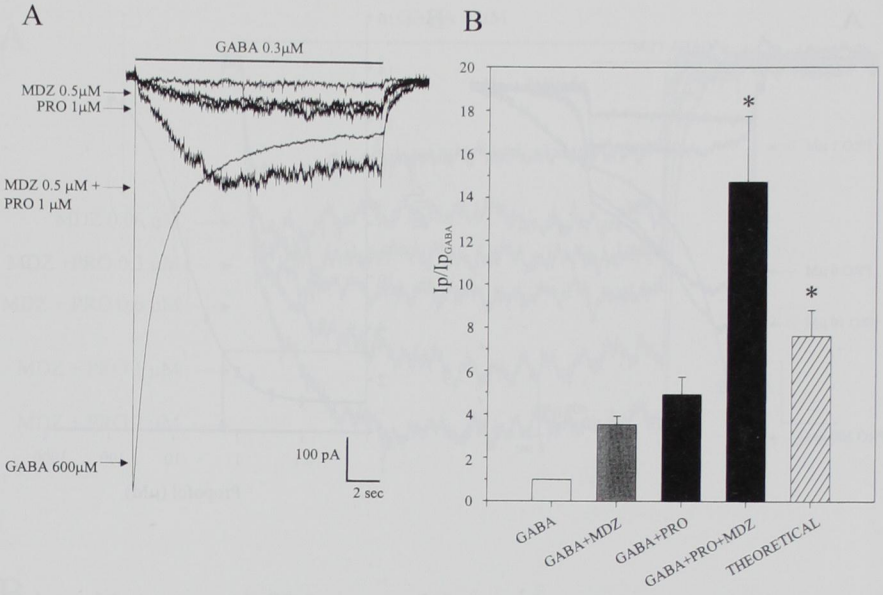

Fig. 2. A supraadditive interaction between midazolam and propofol was apparent when the concentration of γ-aminobutyric acid (GABA) was reduced to 0.3 μM. (*A*) Responses evoked by 0.3 μM GABA, GABA + 0.5 μM midazolam, GABA + 1 μM propofol, and GABA + propofol + midazolam were smaller than the currents evoked by a saturating concentration (600 μM) of GABA. (*B*) The peak currents for each group were normalized to the peak GABA (0.3 μM) current. The theoretical additive response was significantly less than the measured responses to midazolam and propofol ($P < 0.05$, Student's *t* test, n = 4).

propofol was again additive at this lower concentration of propofol ($P > 0.05$; Student's *t* test). Because the enhancement of GABA-evoked current by anesthetics is greatest at low concentrations of GABA, in the next series of experiments, the concentration of GABA was reduced from 3 μM to 0.3 μM. Interestingly, a supraadditive interaction was observed between 0.5 μM midazolam and 1 μM propofol (fig. 2) in the presence of this lower concentration of GABA. GABA-evoked currents were enhanced by midazolam, propofol, and midazolam plus propofol by a factor of 3.54 ± 0.39, 4.93 ± 0.80, and 14.73 ± 3.03, respectively (n = 4; $P < 0.05$, Student's *t* test).

Isobolographic Analysis of Propofol and Midazolam

The supraadditive interaction observed between midazolam and propofol in the enhancement of current activated by low concentrations of GABA was further investigated using isobolographic analysis. The concentration of GABA used to elicit the control response was increased from 0.3 μM to 1 μM because the small current activated by the lower concentration of GABA was difficult to resolve from the background noise. GABA at a dose of 1 μM activated approximately 9% of the maximum response evoked by a saturating concentration of GABA. The peak amplitude of current activated by 1 μM GABA and of various concentrations of propofol (0.1 to 100 μM) was measured, and the concentration–response curve for propofol-induced enhancement of GABA-evoked current was constructed (fig. 3A).

The maximal increase of current was produced by 100 μM propofol, which enhanced the response by a factor of 12.6 ± 3.4 (n = 9, fig. 3B). Thus, the current activated by a low concentration of GABA (EC_9) was increased 12.6 times and had an amplitude similar to that evoked by a saturating concentration of GABA. The similar maximum response suggests that the same population of receptors is activated by GABA (EC_{100}) and GABA (EC_9) plus propofol. The concentration of propofol that produced 50% of the maximal increase and the calculated Hill coefficient were 7.12 ± 1.34 μM and 1.27 ± 0.15 (n = 9), respectively (fig. 3B). The concentration of propofol that increased the amplitude of the control response by a factor of three was 2.42 ± 0.43 μM.

Midazolam also produced a concentration-dependent increase in the amplitude of GABA (1 μM)-evoked currents (fig. 4A). The concentration–response relation for midazolam-modulated current is illustrated in figure 4B. The EC_{50} and Hill coefficients for potentiation of GABA-evoked current were 0.10 ± 0.03 μM and 1.18 ± 0.18, respectively. The maximal increase observed with midazolam was considerably less than that observed with propofol, presumably because midazolam does not directly activate the receptor. GABA-evoked currents were maximally increased by a factor of 4.25 ± 0.55 (n = 9). However, in two cells, the maximal enhancement of the GABA-evoked responses by midazolam failed to reach the desired effect level (2.25 and 2.37, respectively), and data from these two cells were not included in further analysis. The concentration of midazolam that enhanced

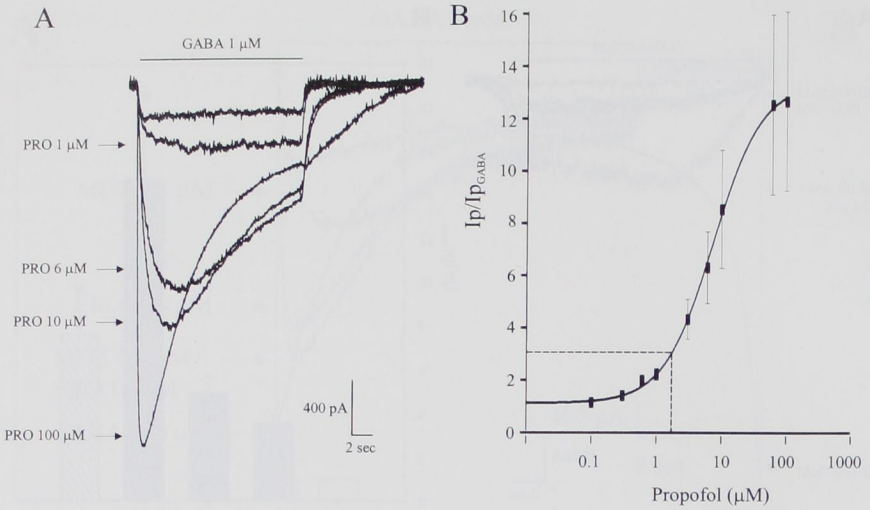

Fig. 3. Propofol potentiation of the γ-aminobutyric acid (GABA)-evoked (1 μM) response. (*A*) Responses evoked by 1 μM GABA were potentiated by increasing concentrations of propofol (1 to 100 μM). (*B*) Propofol dose dependently potentiated GABA-evoked currents. The EC_{50} and Hill coefficient for midazolam potentiation were 7.21 ± 1.34 μM and 1.27 ± 0.15, respectively (n = 9). The maximum potentiation was 1,264 ± 341%. The concentration of propofol that produced a 300% effect level, in the presence of 1 μM GABA, was 2.42 ± 0.43 μM (n = 9). The dashed line on the concentration–response curve indicates the dose that produced a 300% effect level.

GABA-evoked responses by a factor of three was 0.18 ± 0.08 μM (n = 7, fig. 4B).

To evaluate the interaction between midazolam and propofol and to complete the isobolographic analysis, a low concentration of midazolam (0.06 μM) was selected. Currents were recorded in the presence of 1 μM GABA, 0.06 μM midazolam, and various concentrations of propofol (0.06 to 6 μM; fig. 5A). The concentration of propofol necessary to increase the control response by a factor of three in the presence of 0.06 μM midazolam was determined from individual concentration–response curves constructed for current from each cell (fig. 5B).

The concentrations of midazolam and propofol alone that produced the increase in current to the effect level were plotted on the x and y axes of the isobologram, respectively (fig. 6), and a straight line was drawn between these points to form the isobol of additivity. The interpolated concentrations of propofol that caused the threefold increase in current recorded in the presence of 1 μM GABA and 0.06 μM midazolam were then plotted on the isobologram. In all cells tested (n = 9), the concentration of propofol that produced the effect level was lower than the concentration predicted by the isobol of additivity. Furthermore, the average concentration of propofol (0.63 ± 0.20 μM, n = 9) was significantly lower than the predicted additive concentration (1.63 ± 1.22 μM, n = 9; $P < 0.05$, Student's *t* test), showing that propofol and midazolam interact synergistically to enhance GABA (1 μM)-evoked currents.

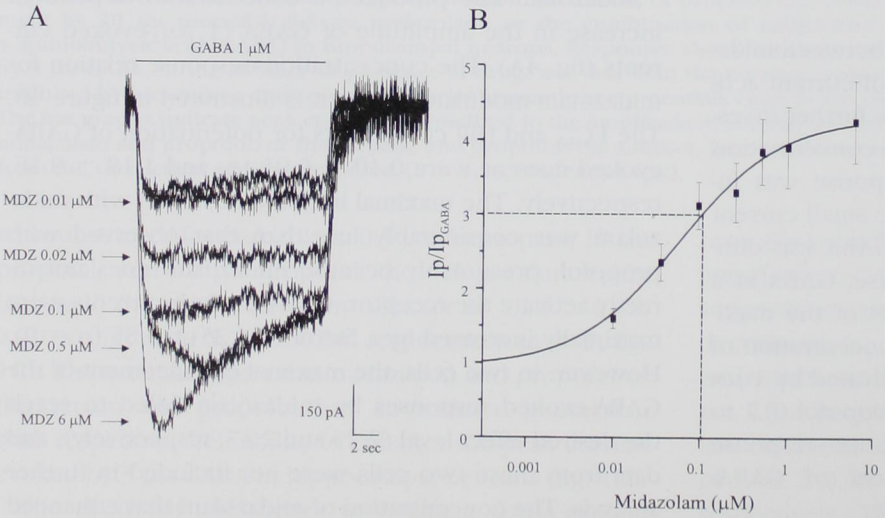

Fig. 4. Midazolam potentiation of the γ-aminobutyric acid (GABA)-evoked (1 μM) response. (*A*) Responses evoked by 1 μM GABA were potentiated by increasing concentrations of midazolam (0.1 to 6 μM). (*B*) Midazolam dose dependently potentiated GABA-evoked currents. The EC_{50} and Hill coefficient for midazolam potentiation were 0.102 ± 0.034 μM and 1.182 ± 0.182, respectively (n = 9). High concentrations of midazolam increased the peak amplitude by 425 ± 55%. The concentration of midazolam that caused the 300% effect level in the presence of 1 μM GABA was 0.182 ± 0.076 μM for seven cells. The dashed line on the concentration–response curve indicates the dose that produced the specified effect level.

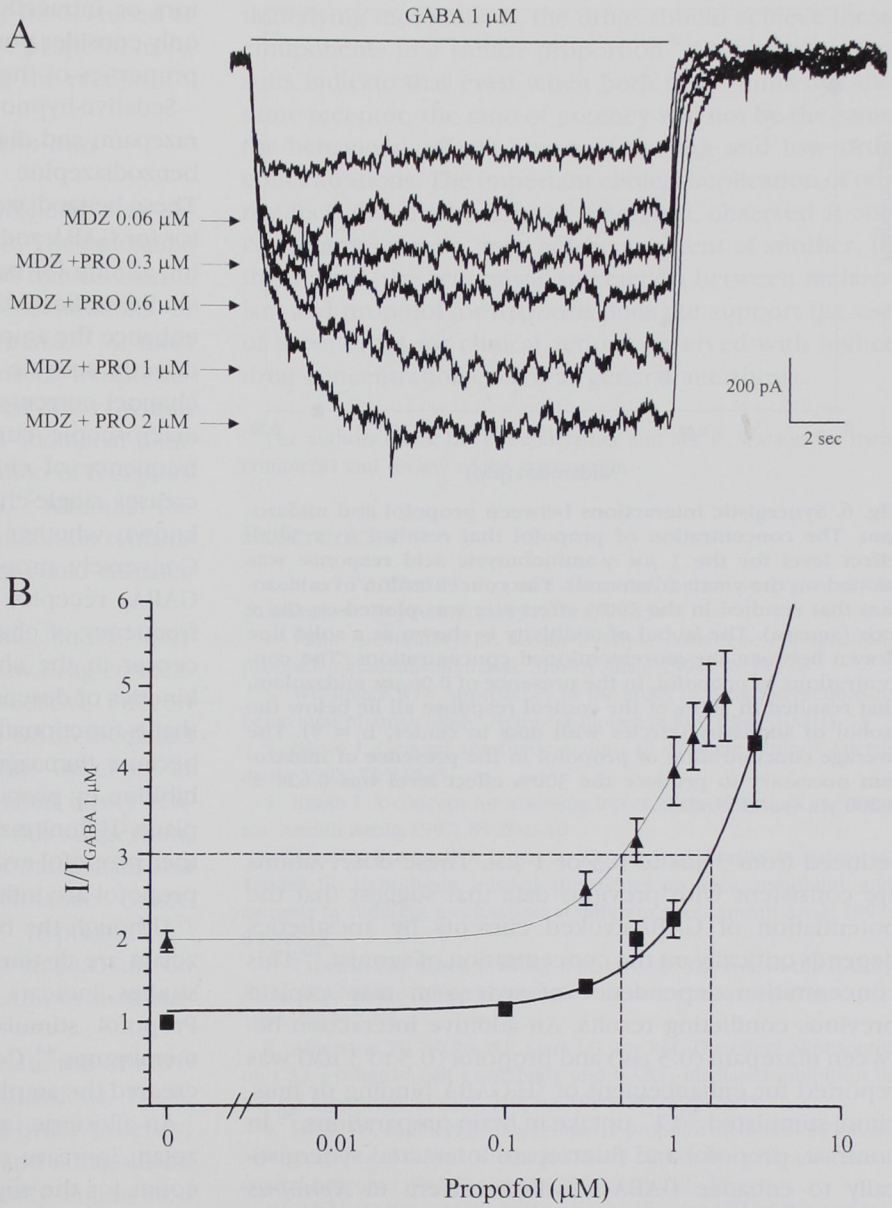

Fig. 5. Propofol enhancement of currents evoked by coapplication of midazolam and γ-aminobutyric acid (GABA). (*A*) Raw traces of propofol (0.06 to 2 μM) potentiation of midazolam (0.06 μM)- and GABA-evoked (1 μM) currents. (*B*) Propofol potentiates GABA-evoked currents in a dose-dependent manner. The concentration of propofol that resulted in a 300% effect level, in the presence of 0.06 μM midazolam and 1 μM GABA, was 0.63 ± 0.20 μM (n = 9). The dashed line on the concentration–response curve represents the concentration of propofol that produced a 300% effect level.

The supraadditive effect of midazolam and propofol was confirmed in a subsequent experiment using the fixed-concentration method. We evaluated the effects of 0.62 μM propofol, 0.6 μM midazolam, and the combination of 0.62 μM propofol and 0.6 μM midazolam on currents activated by 1 μM GABA. Current amplitudes were increased by factors of 2.0 ± 0.04, 1.78 ± 0.1, and 3.34 ± 0.14 (n = 4). The predicted additive response (2.81 ± 0.12) was significantly less than the observed response, confirming a synergistic interaction.

Discussion

In this study, we evaluated the interactions between benzodiazepines and propofol for modulating $GABA_A$ receptors in embryonic mouse neurons. We observed an additive enhancement of GABA (3 μM)-evoked currents by propofol (1 or 10 μM) and midazolam (0.5 μM) in hippocampal neurons. In contrast, a synergistic interaction between midazolam (0.5 μM) and propofol (1 μM) was apparent when the concentration of GABA was

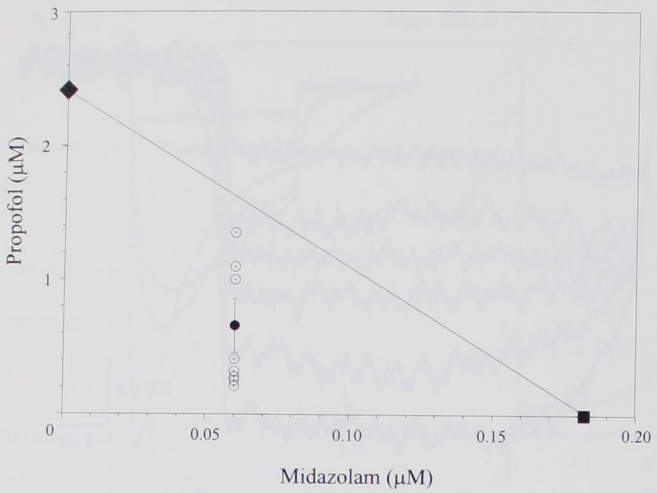

Fig. 6. Synergistic interactions between propofol and midazolam. The concentration of propofol that resulted in a 300% effect level for the 1 μM γ-aminobutyric acid response was plotted on the y axis (diamond). The concentration of midazolam that resulted in the 300% effect size was plotted on the x axis (square). The isobol of additivity is shown as a solid line drawn between the aforementioned concentrations. The concentrations of propofol, in the presence of 0.06 μM midazolam, that resulted in 300% of the control response all lie below the isobol of additivity (circles with dots in center, n = 9). The average concentration of propofol in the presence of midazolam necessary to produce the 300% effect level was 0.624 ± 0.200 μM (solid circle).

reduced from 3 μM to 0.3 or 1 μM. These observations are consistent with previous data that suggest that the potentiation of GABA-evoked currents by anesthetics depends critically on the concentration of agonist.[20] This concentration dependence of synergism may explain previous conflicting results. An additive interaction between diazepam (0.3 μM) and propofol (0.3 to 3 μM) was reported for enhancement of ^{3}H-GABA binding or muscimol-stimulated $^{36}Cl^{-}$ uptake in brain preparations.[30] In contrast, propofol and flurazepam interacted synergistically to enhance GABA-activated current in *Xenopus* oocytes expressing human recombinant $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_2\beta_2\gamma_{2L}$ receptor constructs.[35]

The interactions between midazolam and propofol on $GABA_A$ receptor function are complex, and several possible mechanisms could account for the supraadditive increase in GABA-evoked current. Dissimilar to clinical studies in which pharmacokinetic or pharmacodynamic factors may contribute to the apparent synergism, in our study we assumed that the effective concentration of the drug at the receptor is known and is not influenced by the presence of the other drug. Thus, synergism can be attributed to direct modulation of $GABA_A$ receptor function or functional changes to other unidentified receptors or intracellular signaling pathways. Here we will only consider possible changes to the binding and gating properties of the $GABA_A$ receptor.

Sedative-hypnotic compounds such as midazolam, flurazepam, and diazepam bind to the flumazenil-sensitive benzodiazepine site on the $GABA_A$ receptor.[13,14,26] These benzodiazepines increase the affinity of the receptor for GABA and, reciprocally, GABA increases the binding affinity for benzodiazepines.[36–39] Electrophysiologic studies indicate that benzodiazepines, such as diazepam, enhance the amplitude of whole-cell currents but do not directly activate the receptor.[36,40,41] Studies of single-channel currents indicate that the enhancement of the macroscopic current results from an increase in the frequency of channel opening.[29,42] Diazepam also increases single-channel conductance, although it is not known whether midazolam produces similar effects.[43] Conversely, propofol has at least three distinct effects on $GABA_A$ receptor function, including an increase in the frequency of channel opening,[29,42] activation of the receptor in the absence of GABA, and alteration of the kinetics of desensitization.[9–11,29] Propofol binds at a site that is functionally distinct from the benzodiazepine site, because flumazenil does not effect ^{35}S-TBPS binding inhibition by propofol. Conversely, propofol does not displace ^{3}H-flunitrazepam from cerebral synaptosomes. Neither propofol-evoked currents nor the clinical effects of propofol are influenced by flumazenil.[11,27,34,36,37,42]

Although the binding domains for propofol and midazolam are distinct, radiolabeling and electrophysiologic studies indicate that they are functionally coupled. Propofol stimulated ^{3}H-flunitrazepam binding to rat membranes.[44] Conversely, diazepam and midazolam increased the amplitude of propofol-induced currents.[11,45]

An allosteric interaction, whereby propofol and midazolam increase the binding affinity for GABA, may account for the supraadditive interaction for current activated by 0.1 to 1 μM but not by 3 μM GABA observed in our study. Synergism was no longer apparent when receptors were activated by higher concentrations of GABA, possibly because of the saturation of binding sites. However, 3 μM GABA is less than the EC_{50} value, and the amplitude of current, recorded in the presence or absence of drugs, was less than that activated by 600 μM GABA. Therefore, we favor an explanation that attributes the synergistic interaction to direct activation of the receptor by propofol. Propofol-evoked currents are enhanced by midazolam,[45] therefore the supraadditive interaction between midazolam and propofol could result from midazolam-induced potentiation of the propo-

fol-evoked current. This interaction may be occluded at higher concentrations of GABA, because propofol has a lower potency than GABA for activating the receptor.

Clinical Synergism between Midazolam and Propofol

Synergism between midazolam and propofol was observed only at low concentrations of GABA. The functional significance of this interaction to synaptic transmission is questionable because near-saturating concentrations of GABA are released from the synaptic terminal.[46] At most GABAergic synapses, high concentrations of transmitter rapidly activate current that peaks and then decays within the millisecond time range. However, an additional tonic inhibition can arise from persistent activation of receptors by low ambient concentrations of GABA.[47] Although the functional significance of this persistent inhibition remains to be elucidated, midazolam and propofol could enhance the tonic activation of $GABA_A$ receptors.

Schultz and MacDonald[48] proposed that "subtle" clinical actions of barbiturates observed at low drug concentrations (such as anticonvulsant and sedative effects) might be caused by an augmentation of GABA-mediated inhibition. In contrast, anesthetic actions observed at higher drug concentrations may result from direct activation of the receptor. Consistent with this suggestion, low, clinically relevant concentrations of midazolam and propofol interact synergistically to enhance GABA-evoked currents and also to induce hypnosis.[49–53] Higher concentrations of propofol and midazolam do not interact synergistically to enhance GABA-evoked current or to induce surgical anesthesia.

Various methods have been used to relate the *in vitro* effects of anesthetics to their behavioral actions. These include studies of optical isomers; rank-order potency; drug interactions, including synergism and antagonism; and, more recently, the use of genetically engineered animals. Here we evaluated the interactions between propofol and midazolam in the modulation of $GABA_A$ receptors to account for synergism observed for the induction of hypnosis. It has been postulated that a synergistic interaction implies that the two anesthetics act through distinct mechanisms.[5] Others[54] suggest that an additive interaction is consistent with the drugs acting at independent sites. Here we demonstrate an additive and synergistic interaction between propofol and midazolam for modulating a single target receptor. Thus, drug interactions cannot account for drugs acting at one or more sites. Furthermore, it was postulated that if different clinical actions of the anesthetic have the same underlying mechanisms, the drugs should achieve these components in a similar proportion.[5] However, our results indicate that even when both drugs influence the same receptor, the ratio of potency will not be the same for behavioral effects observed at high and low drug concentrations. The important clinical implication of our results is that the benefits of synergism, observed at one concentration ratio, may not be apparent at another. In this regard, the synergistic interaction between midazolam and propofol for hypnosis does not support the use of these drugs for clinical actions observed with higher drug concentrations, such as general anesthesia.

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References

1. Short TG, Chui PT: Propofol and midazolam act synergistically in combination. Br J Anaesth 1991; 67:539–45

2. Vinik HR, Bradley EL, Kissin I: Triple anesthetic combination: propofol-midazolam-alfentanil. Anesth Analg 1994; 78:354–8

3. Wessinger WD: Approaches to the study of drug interactions in behavioral pharmacology. Neurosci Biobehav Rev 1986; 10:103–13

4. Kissin I: General anesthetic action: an obsolete notion? Anesth Analg 1993; 76:215–8

5. Kissin I: A concept for assessing interactions of general anesthetics. Anesth Analg 1997; 85:204–10

6. Eger EI, Koblin DD, Harris A, Kendig JJ, Pohorille A, Halsey M, Trudell JR: Hypothesis: inhaled anesthetics produce immobility and amnesia by different mechanisms at different sites. Anesth Analg 1997; 84:915–8

7. Tanelian DL, Kosek P, Mody I, MacIvor B: The role of the $GABA_A$ receptor/chloride channel complex in anesthesia. ANESTHESIOLOGY 1993; 78:757–78

8. Alberston TE, Walby WF, Stark LG, Joy RM: The effect of propofol on CA1 pyramidal cell excitability and $GABA_A$-mediated inhibition in the rat hippocampal slice. Life Sci 1996; 58:2397–2407

9. Hales TG, Lambert JJ: The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones. Br J Pharmacol 1991; 104:619–28

10. Hara M, Ikemoto Y: Enhancement by propofol of the gamma-aminobutyric acid A response in dissociated hippocampal pyramidal neurons of the rat. ANESTHESIOLOGY 1994; 81:988–94

11. Hara M, Yoshihisa K, Ikemoto Y: Propofol activates $GABA_A$ receptor-chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat. ANESTHESIOLOGY 1993; 79:781–8

12. Carlson BX, Hales TG, Olsen RW: $GABA_A$ receptors and Anesthesia, Anesthesia Biological Foundations. Edited by Yaksh TL, Lynch III C, Zapol WM, Maze M, Biebuyck JF, Saidman LJ. Philadelphia, Lippincott-Raven Publishers, 1998, pp 259–75

13. Johnston GA: $GABA_A$ receptor pharmacology. Pharmacol Ther 1996; 69:173–98

14. MacDonald RL, Olsen RW: $GABA_A$ receptor channels. Annu Rev Neurosci 1994; 17:569–602

15. Bormann J, Feigenspan A: $GABA_C$ receptors. Trends Neurosci 1995; 18:515–19

16. Kaila K: Ionic basis of $GABA_A$ receptor channel function in the nervous system. Prog Neurobiol 1994; 42:489–537

17. Whiting PJ, McAllister G, Vassilatis D, Bonnert TP, Heavens RP, Smith DW, Hewson L, O'Donnell R, Rigby MR, Sirinathsinghji DJ, Marshall G, Thompson SA, Wafford KA: Neuronally restricted RNA splicing regulates the expression of a novel $GABA_A$ receptor subunit conferring atypical functional properties. J Neurosci 1997; 17:5027–37

18. Davies PA, Hanna MC, Hales TG, Kirkness EF: Insensitivity to anaesthetic agents conferred by a class of GABA(A) receptor subunit. Nature 1997; 385:820–3

19. Lavoie AM, Twyman RE: Direct evidence for diazepam modulation of $GABA_A$ receptor microscopic affinity. Neuropharmacol 1996; 35:1383–92

20. Harris RA, Mihic SJ, Dildy-Mayfield JE, Machu TK: Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition. FASEB J 1995; 9:1454–62

21. Poncer JC, Durr R, Gahwiler BH, Thompson SM: Modulation of synaptic $GABA_A$ receptor function by benzodiazepines in area CA3 of rat hippocampal slice cultures. Neuropharmacol 1996; 35:1169–79

22. Rogers CJ, Twyman RE, Macdonald RL: Benzodiazepine and beta-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurones in culture. J Physiol 1994; 15:69–82

23. Nusser Z, Cull-Candy S, Farrant M: Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. Neuron 1997; 19:697–709

24. Hunkeler W, Mohler H, Pieri L, Polc P, Bonetti EP, Cumin R, Schaffner R, Haefely W: Selective antagonists of benzodiazepines. Nature 1981; 290:514–16

25. Reves JG, Fragen RJ, Vinik HR, Greenblatt DJ: Midazolam: pharmacology and uses. ANESTHESIOLOGY 1985; 62:310–24

26. Fassoulaki A, Sarantopoulos C, Papilas K: Flumazenil reduces the duration of thiopentone but not of propofol anaesthesia in humans. Can J Anaesth 1993; 40:10–12

27. Murayama T, Shingu K, Ogawa T, Tomoda K, Shindo K, Tamai S, Mori K: Fluazenil does not antagonize halothane, thiamylal or propofol anaesthesia in rats. Br J Anaesth 1992; 69:61–4

28. Fan SZ, Liu CC, Yu HY, Chao CC, Lin SM: Lack of effect of flumazenil on the reversal of propofol anaesthesia. Acta Anaesthesiol Scand 1995; 39:299–301

29. Orser BA, Wang LY, Pennefather PS, MacDonald JF: Propofol modulates activation and desensitization of $GABA_A$ receptors in cultured murine hippocampal neurons. J Neurosci 1994; 14:7747–60

30. Concas A, Santoro G, Serra M, Sanna E, Biggo G: Neurochemical action of the general anaesthetic propofol on the chloride ion channel coupled with $GABA_A$ receptors. Brain Res 1991; 542:225–32

31. Plummer JL, Short TG: Statistical modeling of the effects of drug combinations. J Pharmacol Methods 1990; 23:297–309

32. Tallarida RJ, Porreca F, Cowan A: Statistical analysis of drug-drug and site-site interactions with isobolograms. Life Sciences 1989; 45: 947–61

33. Tallarida RJ: Statistical analysis of drug combinations for synergism. Pain 1992; 49:93–7

34. Berenbaum MC: What is synergy? Pharmacol Rev 1989; 41:93–141

35. Reynolds JN, Maitra R: Propofol and flurazepam act synergistically to potentiate $GABA_A$ receptor activation in human recombinant receptors. Eur J Pharm 1996; 314:151–6

36. Costa E, Guidotti A: Molecular mechanisms in the receptor action of benzodiazepines. Ann Rev Pharmacol Toxicol 1979; 19: 531–45

37. Braestrup C, Neilson M: [^{3}H] Propyl β-carboline-3-carboxylate as a selective radioligand for the BZ_1 benzodiazepine receptor subclass. J Neurochem 1981; 37:333–41

38. Braestrup C, Albrechtsen R, Squires RF: High densities of benzodiazepine receptors in human cortical areas. Nature 1977; 269: 702–4

39. Möhler H, Okada T: Benzodiazepine receptor: demonstration in the central nervous system. Science 1977; 198:849–51

40. Choi DW, Farb DH, Fishbach GD: Chlordiazepoxide selectively potentiates GABA conductance of spinal cord and sensory neurons in cell culture. J Neurophysiol 1981; 45:621–31

41. Study RE, Barker JL: Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid response in cultured central neurons. Proc Natl Acad Sci USA 1981; 78:7180–4

42. Peduto VA, Concas A, Santoro G, Biggio G Gessa GL: Biochemical and electrophysiological evidence that propofol enhances GABAergic transmission in the rat brain. ANESTHESIOLOGY 1991; 75:1000–9

43. Eghbali M, Curmi JP, Birnir B, Gage PW: Hippocampal $GABA_A$ channel conductance increased by diazepam. Nature 1997; 388:71–5

44. Prince RJ, Simmonds MA: Propofol potentiates the binding of [^{3}H] flunitrazepam to the $GABA_A$ receptor complex. Brain Res 1992; 596:238–42

45. McAdam, LC, MacDonald, JF, Orser, BA: Midazolam increases the affinity of the $GABA_A$ receptor for propofol. Can J Anaesth 1996; 43:A49

46. Mody I, De Koninck TS, Otis I, Soltez I: Bridging the cleft at the GABA synapses in the brain. Trends Neurosci 1994; 12:517–25

47. Lerma J, Herranz AS, Herras O, Abraira V Martin del rio R: *In vivo* determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. Brain Res 1986; 384:145–55

48. Schultz DW, MacDonald RL: Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: correlation with anticonvulsant and anesthetic actions. Brain Res 1981; 209:177–88

49. McClune S, McKay AC, Wright PM, Patterson CC, Clarke RS: Synergistic interaction between midazolam and propofol. Br J Anaesth 1992; 69:240–5

50. Oxom D, Ferris LE, Harrington E, Orser BA: The effects of midazolam on propofol-induced anaesthesia: propofol dose requirements, mood profiles, and perioperative dreams. Anesth Analg 1997; 85:553–9

51. Heizmann P, Eckert M, Ziegler WH: Pharmacokinetic and bioavailability of midazolam in man. Br J Clin Pharmacol 1983; 16:43S–49S

52. Nilsson A, Tamsen A, Persson P: Midazolam-fentanyl anaesthesia for major surgery. Plasma levels of midazolam during prolonged total intravenous anaesthesia. Acta Anaesthesiol Scan 1986; 30:66–9

53. Kanto J, Gepts E: Pharmacokinetic implications for the clinical use of propofol. Clin Pharmacokinetic 1989; 17:308–26

54. Unkelbach HD Pöch G: Comparison of independence and additivity in drug combinations. Arzneim Forsch Drug Res 1988; 38:1–6