Title

Anesthetic synergy between two n-alkanes

Journal

Veterinary Anaesthesia and Analgesia, 44(3)

ISSN

1467-2987

Authors

Brosnan, Robert J
Fukushima, Fabíola B
Pham, Trung L

Publication Date

2017-05-01

DOI

10.1016/j.vaa.2016.07.003

Peer reviewed

HHS Public Access

Author manuscript

Vet Anaesth Analg. Author manuscript; available in PMC 2018 May 01.

Published in final edited form as:

Vet Anaesth Analg. 2017 May ; 44(3): 577–588. doi:10.1016/j.vaa.2016.07.003.

Anesthetic synergy between two n-alkanes

Robert J Brosnan, **Fabíola B Fukushima**, and **Trung L Pham**

Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA

Abstract

Objective—N-butane and n-pentane each can produce general anesthesia. Both compounds potentiate $GABA_A$ receptor function, but only butane inhibits NMDA receptors. It was hypothesized that butane and pentane would exhibit anesthetic synergy due to their different actions on ligand-gated ion channels.

Study design—Prospective experimental study.

Animals—Four *Xenopus laevis* frogs and 43 Sprague Dawley rats.

Methods—Alkane concentrations for all studies were determined via gas chromatography. Using a *Xenopus* oocyte expression model, standard two-electrode voltage clamp techniques were used to measure NMDA and $GABA_A$ receptor responses *in vitro* as a function of butane and pentane concentrations relevant to anesthesia. The minimum alveolar concentration (MAC) of butane and pentane were measured separately in rats, and then pentane MAC was measured during co-administration of 0.25, 0.50, or 0.75 times MAC of butane. An isobole with 95% confidence intervals was constructed using regression analysis. A sum of butane and pentane that was statistically less than the lower-end confidence bound isobole indicated a synergistic interaction.

Results—Both butane and pentane dose-dependently potentiated $GABA_A$ receptor currents over the study concentration range. Butane dose-dependently inhibited NMDA receptor currents, but pentane did not modulate NMDA receptors. Butane and pentane MAC in rats was 39.4 ± 0.7 and 13.7 ± 0.4 %, respectively. A small but significant ($p < 0.03$) synergistic anesthetic effect with pentane was observed during administration of either 0.50 or 0.75 × MAC butane.

Conclusion—Butane and pentane show synergistic anesthetic effects *in vivo* consistent with their different *in vitro* receptor effects.

Clinical relevance—Findings support the relevance of NMDA receptors in mediating anesthetic actions for some, but not all, inhaled agents.

Correspondence: Robert J Brosnan, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA. rjbrosnan@ucdavis.edu.

Authors' contributions

RJB: study design, conducted the study, collected and analyzed data, wrote the manuscript; FBF & TLP: conducted the study, collected data, reviewed the manuscript.

Keywords

GABA-A receptor; inhaled anesthetics; isobologram; mechanism of action; minimum alveolar concentration; NMDA receptor

Introduction

One of the unique features of volatile anesthetics is their ability to alter function of a large and phylogenetically diverse number of cell protein targets. Instead of acting through a single receptor, volatile anesthetics probably cause immobility in animals primarily by positively modulating inhibitory ion channels and negatively modulating excitatory ion channels within the spinal cord (Sonner et al. 2003; Brosnan 2013; Steffey et al. 2015).

Criteria required for relevance of a protein to anesthetic mechanisms of action include presence of the receptor at anatomic sites relevant to anesthetic action, qualitative modulation of that receptor in a manner that plausibly reduces neuronal excitability, and quantitative modulation of that receptor in a concentration-dependent manner over a pharmacologically-relevant range (Franks 2006). Inhibitory γ-aminobutyric acid type A ($GABA_A$) receptors and excitatory *N*-methyl-D-aspartate (NMDA) receptors are present throughout the central nervous system (CNS), including the spinal cord, and are potentiated or inhibited, respectively, by volatile anesthetics at concentrations around the minimum alveolar concentration (MAC) (Mihic et al. 1994; Solt et al. 2006; Brosnan & Pham 2011). Given these *in vitro* electrophysiology responses, $GABA_A$ and NMDA receptors seem plausible molecular targets for inhaled anesthetic action.

In vivo pharmacologic responses further support the relevance of these two targets. Immobilizing effects of inhaled anesthetics are primarily mediated via effects on the spinal cord, not the brain (Antognini & Schwartz 1993). Accordingly, administration of the NMDA receptor antagonist dizocilpine causes a greater reduction of isoflurane MAC in rats when administered spinally, next to the anatomic site of action responsible for immobility, than when administered either intracerebroventricularly or intravenously (Stabernack et al. 2003). Furthermore, when either $GABA_A$ or glycine receptors are antagonized respectively in isoflurane- or sevoflurane-anesthetized rats, there is increased NMDA receptor antagonism by the volatile anesthetic at MAC, suggesting that greater suppression of glutamatergic excitability might compensate for loss of inhibitory channel contributions to immobility (Brosnan 2011; Brosnan & Thiesen 2012). Volatile agents that can no longer potentiate $GABA_A$ receptors are nonimmobilizers (Mihic et al. 1994). Results from rat studies comparing isoflurane MAC-sparing effects of lumbar *versus* intracerebroventricular infusions of the antagonist picrotoxin indicate that both spinal and supraspinal potentiation of $GABA_A$ receptors could contribute to immobilizing potency (Zhang et al. 2001).

Quintessential evidence for receptor relevance would be demonstration of anesthetic resistance using animal models in which the drug target has been either substituted by an anesthetic-resistant mutant protein or been removed completely. Unfortunately, this most important criterion remains unsatisfied. Mice with the knock-in N256M β_3 subunit mutation are resistant to the amnestic effects of isoflurane, but the immobilizing potency of isoflurane

was increased in one study (Lambert et al. 2005) and unchanged in another (Rau et al. 2011). Mice with a knock-out mutation of the GluRε1 subunit of the NMDA receptor do have increased isoflurane MAC, but this is an indirect result of increased CNS excitation by monoamine neurotransmitters rather than a direct result of fewer NMDA receptor targets in the CNS (Petrenko et al. 2010).

NMDA receptor modulation by various hydrocarbons exhibits a cut-off effect that is associated with the molar water solubility of the hydrocarbon. Compounds having a calculated molar water solubility less than approximately 1.1mM at 25°C do not affect NMDA receptor function, even at saturated aqueous phase concentrations (Brosnan & Pham 2014). Accordingly, among the straight-chain alkanes, butane has a 1.4 mM aqueous solubility and potentiates $GABA_A$ receptor currents and inhibits NMDA receptor currents. Pentane has a 0.43mM aqueous solubility and potentiates $GABA_A$ receptor currents but does not affect NMDA receptor currents (Brosnan & Pham 2014).

Despite their different molecular actions, both butane gas and pentane vapor are general anesthetics (Liu et al. 1993), and both are examples of n-alkanes. The conventional volatile anesthetics chloroform and halothane are also examples alkanes; however, the respective methane and ethane structures are polyhalogenated to reduce flammability. Although drugs that alter function of a single protein through different mechanisms may produce additive effects in combination (Brosnan & Pham 2011), anesthetics that work though completely different protein targets generally exhibit synergistic effects when combined *in vivo* (Hendrickx et al. 2008). If indeed NMDA receptor inhibition contributes to anesthetic immobility, then butane and pentane—both n-alkanes that differ by only by one carbon in length—are hypothesized to combine synergistically at MAC.

The aims of this study were to measure: the dose-responses of butane and pentane on $GABA_A$ and NMDA receptors expressed in frog oocytes, the MAC of butane and pentane in rats, and the concentrations at MAC during co-administration of butane and pentane in rats.

Materials and methods

Receptor expression in frog oocytes

Xenopus laevis frogs were surgically ovariectomized using a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Ovaries were incubated in 0.2% Type I collagenase (Worthington Biochemical Corp., NJ, USA) to defolliculate oocytes which were washed and stored in fresh and filtered modified Barth's solution composed of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 20 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)2$, 0.41 mM $CaCl_2$, 5 mM sodium pyruvate, gentamycin, penicillin, streptomycin, and corrected to pH=7.4. All salts and antibiotics were ACS grade (Fisher Scientific, PA, USA).

Clones used were provided as a gift from Dr RA Harris (University of Texas, Austin, USA) and were sequenced and compared with references in the National Center for Biotechnology Information database to confirm the identity of each gene. $GABA_A$ receptors were expressed using clones for the human $GABA_A$ α_1 and the rat $GABA_A$ β_2 and $_{2s}$ subunits in pCIS-II

vectors. Approximately 0.25–1 ng total plasmid mixture containing α_1, β_2, or $_2$ genes in a respective ratio of 1:1:10 was injected intranuclearly through the oocyte animal pole and studied 2–4 days later. These plasmid ratios ensured incorporation of the subunit into expressed receptors, as confirmed via receptor potentiation to 10 μM chlordiazepoxide or insensitivity to 10 μM zinc chloride during co-application with GABA. In separate oocytes, glutamate receptors were expressed using rat NMDA NR1 clones in a pCDNA3 vector and rat NMDA NR2A clones in a Bluescript vector. RNA encoding each subunit was prepared using a commercial transcription kit (T7 mMessage mMachine; Ambion Inc., TX, USA) was mixed in a 1:1 ratio, and 1–10 ng of total RNA was injected into oocytes and studied 1–2 days later. Oocytes injected with similar volumes of water served as controls.

Alkane dose effects on receptor electrophysiology

Oocytes were studied in a 250 μL linear-flow perfusion chamber with solutions administered by syringe pump at 1.5 mL minute^{-1} with gastight glass syringes and foil-wrapped polytetrafluoroethylene tubing. Whole cell currents were studied using standard two-electrode voltage clamping techniques at holding potentials of −80mV or −60mV for oocytes expressing $GABA_A$ or NMDA receptors, respectively.

Methods for measuring whole-cell $GABA_A$ receptor currents have been described (Brosnan et al. 2006; Yang et al. 2007a, b; Brosnan & Pham 2014). Pairs of identical concentrations of hydrocarbon mixtures were prepared in glass syringes containing Frog Ringer's (FR) solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES in 18.2 MΩ H_2O, filtered and adjusted to pH = 7.4) or frog Ringer's + agonist (FR-GABA; FR plus 20–40μM, 4-aminobutanoic acid, equal to an $EC_{10–20}$). After FR perfusion for 5 minutes, oocytes were exposed to 30 seconds of FR-GABA followed by another 5 minutes FR washout; this was repeated until stable $GABA_A$ receptor-elicited peaks were obtained. Next, the oocyte chamber was perfused with FR containing the study n-alkane for 2 minutes followed by a 30 seconds perfusion with an equal concentration of the same n-alkane dissolved in FR-GABA solution. FR was next perfused for 5 minutes to allow hydrocarbon washout, and oocytes were finally perfused with FR-GABA for 30 seconds to confirm return of currents to within 10% of the initial baseline response.

Methods for measuring whole-cell NMDA receptor currents have been described (Brosnan et al. 2006; Yang et al. 2007b; Brosnan & Pham 2008, 2011, 2014). Baseline perfusion solutions were the same as for $GABA_A$ with the substitution of equimolar $BaCl_2$ for calcium salts and the addition of 0.1 mM EGTA; this constituted barium frog Ringer's solution (BaFR). Agonist solutions for NMDA studies also contained 0.1 mM glutamate (E) and 0.01 mM glycine (G) to constitute a BaFREG solution that produced a NMDA receptor current ≥ EC99. The perfusion protocols for baseline current measurements, alkane washin, alkane +agonist response measurements, and alkane washout and baseline verification were identical to those previously described for $GABA_A$ receptor currents.

Gas chromatography measurements

Concentrations of butane gas and pentane vapor were measured using a gas chromatograph (Clarus 500; Perkin Elmer, MA, USA) with direct sample injections onto a 1829 mm long, 6

mm diameter, packed SF-96 column (WR Grace & Co., MD, USA) for separation using an isothermal protocol with a 50°C injector, 100°C oven, and 150°C flame ionization detector temperatures. Hydrogen and air flows were held constant at 35 mL minute^{-1} and 350 mL minutes^{-1} flowrates, respectively during each 5 minute sample run. Helium flow rate was 35 mL minute^{-1} for the first 24 seconds and then reduced to 5 mL minute^{-1} for the remainder of the sample run time. The resulting retention times for butane and pentane samples were 1.50 minutes and 2.40 minutes, respectively.

Prior to all studies, the gas chromatograph was calibrated against multiple flasks of butane or pentane standards that encompassed the range of concentrations studied and that were prepared using Ideal Gas Laws (Barratt 1981). To determine alkane concentrations in oocyte perfusate solutions, the Ostwald saline-gas partition coefficients for butane and pentane were measured in frog Ringer's solution at 23°C using a glass syringe headspace equilibration method previously described for measurement of ether anesthetic distribution constants (Soares et al. 2012). These partition coefficients were then used to calculate aqueous hydrocarbon concentrations of chamber perfusates aspirated into a glass syringe and equilibrated with an air headspace that was analyzed by gas chromatography.

Alkane minimum alveolar concentration in rats

A total of 43 male Sprague-Dawley rats were purchased (Harlan Laboratories, CA, USA) and studied with the approval of the University of California, Davis Institutional Animal Use and Care Committee (Table 1). Animals were fed a commercial rat chow and were housed in a vivarium with a 12 hour light-dark cycle.

All studies were conducted entirely within a ducted fume hood because the gas mixtures are potentially explosive. Rats were placed in separate stoppered acrylic cylinders through which a minimum of 1 L minute^{-1} fresh gas flowed through a port on the proximal end, and a coaxial sampling port extending to the rat's nose was used to sample inspired gas concentrations into a 5 mL glass syringe. The rat's tail was placed through one port on the distal end and the junction sealed, and a passive scavenging hose from within the fume hood was connected to a second distal port. Once immobilized with the study agent(s), 10 mL kg^{-1} of a 0.9% NaCl solution was administered subcutaneously between the scapulae and a thermistor probe traversing the distal stopper was placed in the rectum (the electronic digital monitor was located outside of the fume hood). Body temperature was maintained at 37–38 °C using microwavable gel packs placed outside the acrylic cylinder as needed. Heart rate (HR) and oxygen saturation (SpO_2) were intermittently measured in each animal prior to testing using a pulse oximeter (MouseOx; Starr Life Sciences Corp., PA, USA). Respiratory rate (f_R) was measured by counting thoracic excursions. Rats were euthanized at the end of each experiment using 1 mL kg^{-1} of a saturated KCl solution administered intravenously during deep anesthesia with the study agent(s).

For butane-only experiments, anesthesia was induced with butane in oxygen in separate cylinders with separate two-stage regulators to supply flowmeters with separate flash arrestors. These were connected via a Y-adaptor to a 1 meter silicone convoluted hose to generate turbulent flow over a sufficient length to ensure complete gas mixing before reaching the acrylic animal cylinders. Butane concentrations within the rat cylinder were

measured every 7–8 minutes using gas chromatography with calibration performed prior to each study using standard gas mixtures prepared using ideal gas laws. After 30 minutes equilibration at a constant butane concentration, the rat tail was clamped with an alligator clip for 1 minute. If movement (or no movement) was observed, the butane flowmeter dial was increased (or decreased) in order to increase (or decrease) the butane concentration by 10% or less. After 30 minutes re-equilibration, the tail was clamped again to test for movement. If no movement (or movement) was observed, the butane concentration was decreased (or increased) by 10% or less, and the rat was re-equilibrated and tested for movement as previously described. Butane MAC was defined as the average of the highest butane concentration that allowed movement and the subsequent lowest butane concentration that prevented movement in response to the noxious stimulus. For each experiment, butane MAC was measured in triplicate.

Pentane MAC in rats was measured under similar conditions and in anesthesia chambers identical to those described for butane MAC. Anesthesia was induced with 15–20% pentane in oxygen using a copper kettle vaporizer. The vaporizer was pre-chilled prior to use, filled with cooled n-pentane (Sigma-Aldrich Corporation, MO), and oxygen was administered at a constant flow (ranging between 230 and 400 mL minute^{-1}) until thermal equilibrium with the ambient environment (22–23 °C) resulted in a constant liquid anesthetic concentration at 15–17 °C, depending on the fresh gas flow rate used for the experiment. Bypass (non-vaporizer) oxygen diluent flow equaled ≥ 1 L minute^{-1} per cylinder. Pentane concentrations within the rat cylinder were measured every 7–8 minutes using gas chromatography with calibration performed prior to each study using standard gas mixtures prepared using ideal gas laws. After 30 minutes equilibration at a constant pentane concentration, response to noxious tail clamp stimulation was tested in a manner identical to that previously described for n-butane. If the rat moved, pentane concentration was increased by 10% or less; if the rat did not move, pentane concentration was decreased by 10% or less. As with the initial measurement, animals were equilibrated at each new constant pentane concentration for 30 minutes before re-testing. For each experiment, pentane MAC was measured in triplicate.

Alkane additivity studies in rats

Breathing gases were supplied by two separate sources that were attached to a Y-connector from which a 1 meter convoluted silicone hose delivered the mixed gas to the acrylic animal cylinders. The first gas source was supplied by the butane gas cylinder connected to a flowmeter with a flash arrestor. The second source was supplied by the common gas outlet from a pentane-filled copper kettle anesthetic machine. The anesthesia apparatus and animal instrumentation and monitoring was otherwise identical to that described for prior studies with either butane or pentane alone.

Butane was administered at one of three doses: 25%, 50%, or 75% of the previously determined butane MAC and held constant by controlling the butane flowmeter and anesthetic machine bypass oxygen flowmeter and maintaining a minimum total gas flow of 1 L minute^{-1} cylinder^{-1}. The copper kettle flowmeter was adjusted to deliver the balance of the MAC for pentane, assuming additivity, and alkane concentrations were maintained constant for 30 minutes of equilibration and confirmed every 7–8 minutes using gas

chromatography. The tail was then clamped with an alligator clip in a manner identical to that previously described. If movement was observed, the pentane concentration was increased by 10% or less of the current concentration (while maintaining a constant MAC-fraction of butane), and after 30 minutes re-equilibration, the tail was re-clamped to test for movement. If no movement was observed, the pentane concentration was decreased by 10% or less (while maintaining a constant butane concentration), and the rat was re-equilibrated and tested for movement as previously described. The single pentane MAC in the presence of a constant butane MAC-fraction was calculated in a manner identical to that for butane or pentane alone.

Statistical analysis

Whole cell electrophysiology responses were expressed as percent normalized currents for receptor potentiation, $I_p = 100 \cdot \frac{I_a - I_b}{I_b}$, and for NMDA receptor inhibition, $I_i = 100 \cdot \frac{I_b - I_a}{I_b}$, where I_a and I_b are the alkane current and baseline current, respectively. All *in vitro* and *in vivo* data were summarized as mean ± standard error of the mean (SEM).

MAC values for butane and pentane delivered as single agents modeled (STATA; StataCorp LP, TX, USA) using linear regression with data clustered by individual rat and with robust standard errors calculated by Huber-White sandwich estimators (White 1980; Williams 2000). The model intercept with butane as the dependent regression variable equaled butane MAC, and the model intercept with pentane as the dependent regression variable equaled pentane MAC. These collinear regression lines defined the additive isobole. The 95% confidence intervals for the butane and pentane MAC values were then calculated, and the lower- and upper-bound values were used to construct regression lines that formed a 95% confidence interval around the additivity isobole.

For mixtures of alkanes, the null hypothesis was: $\frac{[butane]}{MAC_{butane}} + \frac{[pentane]}{MAC_{pentane}} \geq 1$, using the lower bound MAC value of the 95% confidence interval for each agent in the denominator. One-sided Student's t-tests were used to detect deviations from additivity (rejection of the null hypothesis) when $p < 0.05$. A synergistic interaction was defined by a sum of butane and pentane MAC fractions that were significantly less than 1 for the lower-bound of the 95% confidence interval isobole.

Results

Oocyte studies were conducted at a room temperature of 23°C. For frog Ringer's solutions at this temperature, the Ostwald liquid-gas partition coefficient was 0.068 ± 0.003 for butane and 0.046 ± 0.007 for pentane. Examples of electrophysiographic tracings from two-electrode voltage clamp studies of oocytes expressing $GABA_A$ receptors or NMDA receptors with and without dissolved butane are shown in Figure 1. Electrophysiologic responses were qualitatively and quantitatively similar for $GABA_A$ receptors exposed to equipotent doses of pentane.

In oocyte voltage clamp studies, butane and pentane both dose-dependently increased normalized $GABA_A$ receptor currents (Fig. 2). Compared with baseline, $GABA_A$ currents

were potentiated approximately 30% during perfusion with 10% of an atmosphere butane (267 μM butane) and were potentiated approximately 175% during perfusion with 40% of an atmosphere butane (1.07 mM butane). $GABA_A$ receptor currents were also increased by about 30% during exposure to 4% pentane (72 μM) and by about 375% during exposure to 14% pentane (253 μM). Butane also dose-dependently inhibited NMDA receptor currents by nearly 20% and 45% at concentrations of 10% and 40%, respectively (Fig. 3). NMDA receptor currents were unchanged when exposed to pentane, even at saturated aqueous phase concentrations of 0.53mM (McAuliffe 1966) that are lethal *in vivo* (Fühner 1921).

The physiologic responses during n-alkane inhalation were characterized by tachycardia, and HR was significantly higher when butane was administered alone *versus* anesthesia with n-alkane mixtures or with pentane alone (Table 1). Anesthesia with either agent was associated with tachypnea without dyspnea; f_R were significantly higher during exposure to 0.5–1.0 butane MAC fractions (F_{MAC}) compared with responses with 0.25 butane F_{MAC} or when only pentane was administered. Tachypnea observed in many rats was not accompanied by either low SpO_2 values or hyperthermia.

The MAC of butane alone was 39.4% of an atmosphere at sea level, and the MAC of pentane alone was 13.7% of an atmosphere. During administration of 25% butane F_{MAC}, pentane concentrations were changed an average of 7.8% for MAC determinations, and the pentane EC_{50} approximately equaled 75% pentane F_{MAC}. Hence, at this alkane ratio, the sum of the F_{MAC} values for butane and pentane were not statistically different from 1 (Table 1), and a plot of this point lies on the isobologram line of additivity (Fig. 4).

During administration either 50% or 75% butane MAC fraction (F_{MAC}), pentane concentrations were changed an average of 7.5% for the determination of MAC. The sum of nalkane F_{MAC} values during administration of 50% or 75% butane F_{MAC} were respectively 0.951 ± 0.009 and 0.938 ± 0.01 (mean ± SEM); these values deviated significantly from the additivity isobole (Fig. 4). If the alkanes behaved additively, 6.78% of an atmosphere pentane would have been required to achieve 1 MAC in rats breathing 19.9% of an atmosphere butane. However, these animals required only 6.10% of an atmosphere pentane, a 10% reduction from the predicted additive value. Even greater synergy was evident during administration of 29.3% butane. At this higher concentration, the predicted pentane MAC was 3.51% of an atmosphere but the measured pentane MAC was 2.67% of an atmosphere, a reduction of 24% from the predicted value.

Discussion

Butane and pentane are dose-dependent $GABA_A$ receptor agonists at concentrations near MAC, but only butane inhibits NMDA receptors at similar concentrations. Both agents are general anesthetics that exhibit synergy when administered in combinations for which butane $F_{MAC} \geq 50\%$. To our knowledge, pentane is the first volatile inhaled anesthetic identified to be selective against NMDA receptor modulation.

Normal alkane MAC values in rats have been previously measured. In a comparison of data in the present study *versus* those of Liu et al.(1993), butane MAC was 39.3% of an

atmosphere *versus* 34.5% of an atmosphere, and pentane MAC was 13.7% of an atmosphere *versus* 12.7% of an atmosphere. The blood-gas partition coefficients for butane (Liu et al. 1994) and pentane (Perbellini et al. 1985) at 37°C are reported as 0.41 and 0.38, respectively. Hence, blood concentrations of butane and pentane in normothermic rats at MAC in the present study were 6.34 mM and 2.05 mM, respectively.

Results also confirm previous *in vitro* electrophysiology data showing 30% inhibition of NMDA receptor inhibition by 1 MAC of butane (Hara et al. 2002) and no NMDA receptor effects by pentane (Brosnan & Pham 2014). In contrast, findings in the present study at first appear to contradict conclusions from other studies that found 1 × MAC of butane at 150 μM had no effect on $GABA_A$ receptors comprised of similar subunits to those used in the present study (Raines et al. 2001, 2003). However, the butane concentration used in these other studies is actually much less than MAC. Anesthetic potency increases approximately 5% per °C decrease in body temperature (Won et al. 2006), and applying this correction to rat MAC measurements at 37°C reported in the present study, the estimated butane MAC for rats at 23°C is 11.6% of an atmosphere. This butane partial pressure produces a 310 μM butane concentration in the frog Ringer's solution at this same temperature, more than double the previously-assumed MAC concentration. When corrected for the new MAC values, the $GABA_A$ receptor responses to butane dose from previous experiments (Raines et al. 2003) resemble those reported here.

Butane and pentane were synergistic at high butane MAC-fractions, but additive at low butane MAC-fractions. Synergy is explained by differences between molecular actions, eg. butane inhibits NMDA receptors whereas pentane does not. However, butane effects on NMDA receptor currents are concentration-dependent and comparatively small at 0.25 × MAC. Presumably, this mirrors low NMDA receptor binding to butane. Even when anesthetics act through different mechanisms, they can interact additively when there is low fractional receptor occupancy at MAC (Shafer et al. 2008). Hence, the combination of 25% butane plus 75% pentane is additive because NMDA receptors, the molecular target that differs between these two normal alkanes, are minimally bound and modulated at MAC when butane concentrations are low.

Although statistically significant, butane-pentane synergy was quantitatively small. This may reflect the relatively small contribution of butane's NMDA receptor inhibition to immobility owing to its comparatively low efficacy at this receptor at an anesthetic median effective concentration (EC_{50}). Inhaled anesthetics for which glutamatergic transmission is postulated to a play major mechanistic role, such as xenon, nitrous oxide, carbon dioxide and cyclopropane, all inhibit NMDA receptors by 29–42 % at concentrations equivalent to 0.5–1.0 × MAC (Yamakura & Harris 2000; Hara et al. 2002; Ogata et al. 2006; Brosnan & Pham 2008). In contrast, butane produces <20% NMDA receptor inhibition at MAC. Presumably, greater synergy might be predicted for combinations of CO_2-pentane or xenon-pentane than between the two n-alkanes studied here.

NMDA receptor inhibition, if present, contributes to the immobilizing effects of anesthetics. However, NMDA receptor inhibition is not required for volatile anesthetic potency. Pentane does not inhibit NMDA receptors even at concentrations far in excess of MAC, yet remains

an immobilizing agent. Despite these different molecular actions, neither pentane nor butane follow the Meyer-Overton rule (Overton 1991); both are much less potent than predicted by their oil:gas partition coefficients (Taheri et al. 1993; Liu et al. 1994). Therefore, the ability or failure to modulate NMDA receptors cannot alone explain why some agents violate the Meyer-Overton relationship.

NMDA and $GABA_A$ receptor actions differ quantitatively between conventional anesthetics that follow Meyer-Overton and n-alkanes that do not. The conventional volatile anesthetics cause moderate NMDA receptor inhibition but produce strong $GABA_A$ receptor potentiation at MAC (Lin et al. 1992; Hollmann et al. 2001; Ogata et al. 2006). Although most of the conventional gas anesthetics produce weak or no $GABA_A$ receptor potentiation at MAC, they tend to more strongly inhibit NMDA receptors (Yamakura & Harris 2000; Hara et al. 2002). However, both butane and pentane weakly potentiate $GABA_A$ currents at MAC, and only butane weakly inhibits NMDA receptor currents. Perhaps one cause for deviation from the Meyer-Overton relationship could be poor drug inhibitory efficacy at $GABA_A$ receptors in the face of poor or absent NMDA receptor efficacy.

NMDA receptor antagonism is not essential to volatile anesthetic potency. But could absence of NMDA receptor effects have other pharmacodynamic consequences? The potent inhaled anesthetics all cause dose-dependent depression of cardiovascular and respiratory system function (Steffey et al. 2015). NMDA receptors within the hypothalamus, medulla, and thoracic spinal cord mediate sympathetic baroreceptor reflexes, and NMDA receptor antagonists selectively administered at these locations result in attenuation of inotropic, chronotropic, and pressor responses (Gordon 1987; Jung et al. 1991; Soltis & DiMicco 1991; Hong & Henry 1992; Bazil & Gordon 1993). In addition, NMDA receptors stimulate phrenic inspiratory drive and peripheral chemoreceptor hypoxic ventilation, thus NMDA receptors in pontine-medullary neurons help regulate breathing pattern whereas their inhibition results in apneusis (Jung et al. 1991; Connelly et al. 1992; Fung et al. 1994; Chitravanshi & Sapru 1996; Ohtake et al. 1998). It is plausible that differences in NMDA receptor effects underlie the significant differences in HR and f_R in rats anesthetized with butane *versus* pentane. Further comparisons of ventilatory and cardiovascular responses over a range of equipotent anesthetic doses are needed to evaluate this hypothesis.

While the present study has focused on NMDA and $GABA_A$ receptor effects, it is acknowledged that the normal alkanes modulate many other receptors that may contribute to anesthetic action. Butane enhances glycine receptor currents and inhibits nicotinic acetylcholine receptor and voltage-gated sodium channel currents (Elliott et al. 1985; Hara et al. 2002; Raines et al. 2002). Pentane also inhibits nicotinic acetylcholine receptors and can reduce some sodium and potassium currents (Haydon et al. 1979; McKenzie et al. 1995). The absent NMDA receptor contribution during pentane anesthesia is thus likely offset by increased contributions from many other ion channels, not just by $GABA_A$ receptors. Theoretically, synergy between butane and pentane could be caused in part by a non-NMDA molecular mechanism assuming there is another, as yet unidentified, relevant receptor target with a similar molar water solubility cut-off as the NMDA receptor (Brosnan & Pham 2014). Differences in NMDA receptor effects aside, the n-alkanes, like the

conventional anesthetics, interact with a large number of diverse protein targets. Their mechanisms cannot be entirely explained by two selective receptor systems.

In conclusion, NMDA receptor inhibition, if present, contributes to the immobilizing effects of general anesthetics, as evidenced by synergy between an alkane NMDA receptor antagonist (butane) and a similar alkane without NMDA receptor effects (pentane). However, because pentane retains anesthetic potency, NMDA receptor inhibition is not required for a volatile agent to be an immobilizer. Anesthetics without NMDA receptor modulation can produce physiological responses, such as those involving cardiovascular and respiratory system function, which differ from responses produced by less receptor-selective agents. Whether these differences translate into reduced adverse systemic effects during general anesthesia remains to be seen.

Acknowledgments

This work was supported by grant number GM092821-02 from the National Institutes of General Medical Sciences (National Institutes of Health). The authors thank Dr Phil Kass, School of Veterinary Medicine, University of California, Davis for statistical advice.

References

Antognini JF, Schwartz K. Exaggerated anesthetic requirements in the preferentially anesthetized brain. Anesthesiology. 1993; 79:1244–1249. [PubMed: 8267200]

Barratt RS. The preparation of standard gas mixtures. The Analyst. 1981; 106:817–849.

Bazil MK, Gordon FJ. Sympathoexcitation from the rostral ventrolateral medulla is mediated by spinal NMDA receptors. Brain Res Bull. 1993; 31:273–278. [PubMed: 8098257]

Brosnan R, Gong D, Cotten J, et al. Chirality in anesthesia II: stereoselective modulation of ion channel function by secondary alcohol enantiomers. Anesth Analg. 2006; 103:86–91. [PubMed: 16790632]

Brosnan RJ. GABA(A) receptor antagonism increases NMDA receptor inhibition by isoflurane at a minimum alveolar concentration. Vet Anaesth Analg. 2011; 38:231–239. [PubMed: 21492389]

Brosnan RJ. Inhaled anesthetics in horses. Vet Clin North Am Equine Pract. 2013; 29:69–87. [PubMed: 23498046]

Brosnan RJ, Pham TL. Carbon dioxide negatively modulates *N*-methyl-D-aspartate receptors. Br J Anaesth. 2008; 101:673–679. [PubMed: 18791188]

Brosnan RJ, Pham TL. Does anesthetic additivity imply a similar molecular mechanism of anesthetic action at *N*-methyl-D-aspartate receptors? Anesth Analg. 2011; 112:568–573. [PubMed: 21233494]

Brosnan RJ, Pham TL. Hydrocarbon molar water solubility predicts NMDA vs. GABAA receptor modulation. BMC Pharmacol Toxicol. 2014; 15:62. [PubMed: 25410726]

Brosnan RJ, Thiesen R. Increased NMDA receptor inhibition at an increased Sevoflurane MAC. BMC Anesthesiol. 2012; 12:9. [PubMed: 22672766]

Chitravanshi VC, Sapru HN. NMDA as well as non-NMDA receptors mediate the neurotransmission of inspiratory drive to phrenic motoneurons in the adult rat. Brain Res. 1996; 715:104–112. [PubMed: 8739628]

Connelly CA, Otto-Smith MR, Feldman JL. Blockade of NMDA receptor-channels by MK-801 alters breathing in adult rats. Brain Res. 1992; 596:99–110. [PubMed: 1468006]

Elliott JR, Haydon DA, Hendry BM, Needham D. Inactivation of the sodium current in squid giant axons by hydrocarbons. Biophys J. 1985; 48:617–622. [PubMed: 2413918]

Franks NP. Molecular targets underlying general anaesthesia. Br J Pharmacol. 2006; 147(Suppl 1):S72–S81. [PubMed: 16402123]

Fühner H. Die narkotische wirkung des benzins und seiner bestandteile (pentan, hexan, heptan, octan). Biochem Z. 1921; 115:235–251.

Fung ML, Wang W, St John WM. Involvement of pontile NMDA receptors in inspiratory termination in rat. Respir Physiol. 1994; 96:177–188. [PubMed: 7914709]

Gordon FJ. Aortic baroreceptor reflexes are mediated by NMDA receptors in caudal ventrolateral medulla. Am J Physiol. 1987; 252:R628–R633. [PubMed: 2881491]

Hara K, Eger EI 2nd, Laster MJ, Harris RA. Nonhalogenated alkanes cyclopropane and butane affect neurotransmitter-gated ion channel and G-protein-coupled receptors: differential actions on GABAA and glycine receptors. Anesthesiology. 2002; 97:1512–1520. [PubMed: 12459679]

Haydon DA, Kimura JE. Some effects of n-pentane on the capacity and ionic currents of the squid giant axon membrane. J Physiol. 1981; 312:57–70. [PubMed: 6267268]

Hendrickx JF, Eger EI 2nd, Sonner JM, Shafer SL. Is synergy the rule? A review of anesthetic interactions producing hypnosis and immobility. Anesth Analg. 2008; 107:494–506. [PubMed: 18633028]

Hollmann MW, Liu HT, Hoenemann CW, et al. Modulation of NMDA receptor function by ketamine and magnesium. Part II: interactions with volatile anesthetics. Anesth Analg. 2001; 92:1182–1191. [PubMed: 11323344]

Hong YG, Henry JL. Glutamate, NMDA and NMDA receptor antagonists: cardiovascular effects of intrathecal administration in the rat. Brain Res. 1992; 569:38–45. [PubMed: 1351773]

Jung R, Bruce EN, Katona PG. Cardiorespiratory responses to glutamatergic antagonists in the caudal ventrolateral medulla of rats. Brain Res. 1991; 564:286–295. [PubMed: 1687375]

Lambert S, Arras M, Vogt KE, Rudolph U. Isoflurane-induced surgical tolerance mediated only in part by beta3-containing GABA(A) receptors. Eur J Pharmacol. 2005; 516:23–27. [PubMed: 15913600]

Lin LH, Chen LL, Zirrolli JA, Harris RA. General anesthetics potentiate gamma-aminobutyric acid actions on gamma-aminobutyric acidA receptors expressed by Xenopus oocytes: lack of involvement of intracellular calcium. J Pharmacol Exp Ther. 1992; 263:569–578. [PubMed: 1331405]

Liu J, Laster MJ, Taheri S, et al. Is there a cutoff in anesthetic potency for the normal alkanes? Anesth Analg. 1993; 77:12–18. [PubMed: 8317717]

Liu J, Laster MJ, Taheri S, et al. Effect of n-alkane kinetics in rats on potency estimations and the Meyer-Overton hypothesis. Anesth Analg. 1994; 79:1049–1055. [PubMed: 7978425]

McAuliffe C. Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin, and aromatic hydrocarbons. J Phys Chem. 1966; 70:1267–1275.

McKenzie D, Franks NP, Lieb WR. Actions of general anaesthetics on a neuronal nicotinic acetylcholine receptor in isolated identified neurones of Lymnaea stagnalis. Br J Pharmacol. 1995; 115:275–282. [PubMed: 7670729]

Mihic SJ, McQuilkin SJ, Eger EI 2nd, et al. Potentiation of gamma-aminobutyric acid type A receptor-mediated chloride currents by novel halogenated compounds correlates with their abilities to induce general anesthesia. Mol Pharmacol. 1994; 46:851–857. [PubMed: 7969071]

Ogata J, Shiraishi M, Namba T, et al. Effects of anesthetics on mutant *N*-methyl-D-aspartate receptors expressed in Xenopus oocytes. J Pharmacol Exp Ther. 2006; 318:434–443. [PubMed: 16622040]

Ohtake PJ, Torres JE, Gozal YM, et al. NMDA receptors mediate peripheral chemoreceptor afferent input in the conscious rat. J Appl Physiol. 1998; 84:853–861. [PubMed: 9480943]

Overton, CE. Studies of Narcosis. Chapman and Hall; UK: 1991.

Perbellini L, Brugnone F, Caretta D, Maranelli G. Partition coefficients of some industrial aliphatic hydrocarbons (C5–C7) in blood and human tissues. Br J Ind Med. 1985; 42:162–167. [PubMed: 3970880]

Petrenko AB, Yamakura T, Kohno T, et al. Reduced immobilizing properties of isoflurane and nitrous oxide in mutant mice lacking the *N*-methyl-D-aspartate receptor GluR(epsilon)1 subunit are caused by the secondary effects of gene knockout. Anesth Analg. 2010; 110:461–465. [PubMed: 19933527]

Raines DE, Claycomb RJ, Forman SA. Nonhalogenated anesthetic alkanes and perhalogenated nonimmobilizing alkanes inhibit alpha(4)beta(2) neuronal nicotinic acetylcholine receptors. Anesth Analg. 2002; 95:573–577. [PubMed: 12198040]

Raines DE, Claycomb RJ, Forman SA. Modulation of GABA(A) receptor function by nonhalogenated alkane anesthetics: the effects on agonist enhancement, direct activation, and inhibition. Anesth Analg. 2003; 96:112–118. [PubMed: 12505935]

Raines DE, Claycomb RJ, Scheller M, Forman SA. Nonhalogenated alkane anesthetics fail to potentiate agonist actions on two ligand-gated ion channels. Anesthesiology. 2001; 95:470–477. [PubMed: 11506122]

Rau V, Oh I, Liao M, et al. Gamma-aminobutyric acid type A receptor beta3 subunit forebrain-specific knockout mice are resistant to the amnestic effect of isoflurane. Anesth Analg. 2011; 113:500–504. [PubMed: 21813630]

Shafer SL, Hendrickx JF, Flood P, et al. Additivity versus synergy: a theoretical analysis of implications for anesthetic mechanisms. Anesth Analg. 2008; 107:507–524. [PubMed: 18633029]

Soares JH, Brosnan RJ, Fukushima FB, et al. Solubility of haloether anesthetics in human and animal blood. Anesthesiology. 2012; 117:48–55. [PubMed: 22510863]

Solt K, Eger EI 2nd, Raines DE. Differential modulation of human *N*-methyl-D-aspartate receptors by structurally diverse general anesthetics. Anesth Analg. 2006; 102:1407–1411. [PubMed: 16632818]

Soltis RP, DiMicco JA. GABAA and excitatory amino acid receptors in dorsomedial hypothalamus and heart rate in rats. Am J Physiol. 1991; 260:R13–R20. [PubMed: 1671541]

Sonner JM, Antognini JF, Dutton RC, et al. Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. Anesth Analg. 2003; 97:718–740. [PubMed: 12933393]

Stabernack C, Sonner JM, Laster M, et al. Spinal *N*-methyl-D-aspartate receptors may contribute to the immobilizing action of isoflurane. Anesth Analg. 2003; 96:102–107. [PubMed: 12505933]

Steffey, EP., Mama, KR., Brosnan, RJ. Inhalation anesthetics. In: Grimm, KA.Lamont, LA.Tranquilli, WJ., et al., editors. Lumb and Jones' Veterinary Anesthesia and Analgesia. 5. John Wiley & Sons, Inc; USA: 2015. p. 237-331.

Taheri S, Laster MJ, Liu J, et al. Anesthesia by n-alkanes not consistent with the Meyer-Overton hypothesis: determinations of the solubilities of alkanes in saline and various lipids. Anesth Analg. 1993; 77:7–11. [PubMed: 8317750]

White H. A heteroskedasticity-consistent covariance matrix estimator and a direct test for heteroskedasticity. Econometrica. 1980; 48:817–830.

Williams RL. A note on robust variance estimation for cluster-correlated data. Biometrics. 2000; 56:645–646. [PubMed: 10877330]

Won A, Oh I, Brosnan R, et al. Hypothermia decreases ethanol MAC in rats. J Anesth. 2006; 20:247–250. [PubMed: 16897251]

Yamakura T, Harris RA. Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels. Comparison with isoflurane and ethanol. Anesthesiology. 2000; 93:1095–1101. [PubMed: 11020766]

Yang L, Milutinovic PS, Brosnan RJ, et al. The plasticizer di(2-ethylhexyl) phthalate modulates gamma-aminobutyric acid type A and glycine receptor function. Anesth Analg. 2007a; 105:393–396. [PubMed: 17646496]

Yang L, Zhao J, Milutinovic PS, et al. Anesthetic properties of the ketone bodies beta-hydroxybutyric acid and acetone. Anesth Analg. 2007b; 105:673–679. [PubMed: 17717222]

Zhang Y, Stabernack C, Sonner J, et al. Both cerebral GABA(A) receptors and spinal GABA(A) receptors modulate the capacity of isoflurane to produce immobility. Anesth Analg. 2001; 92:1585–1589. [PubMed: 11375851]

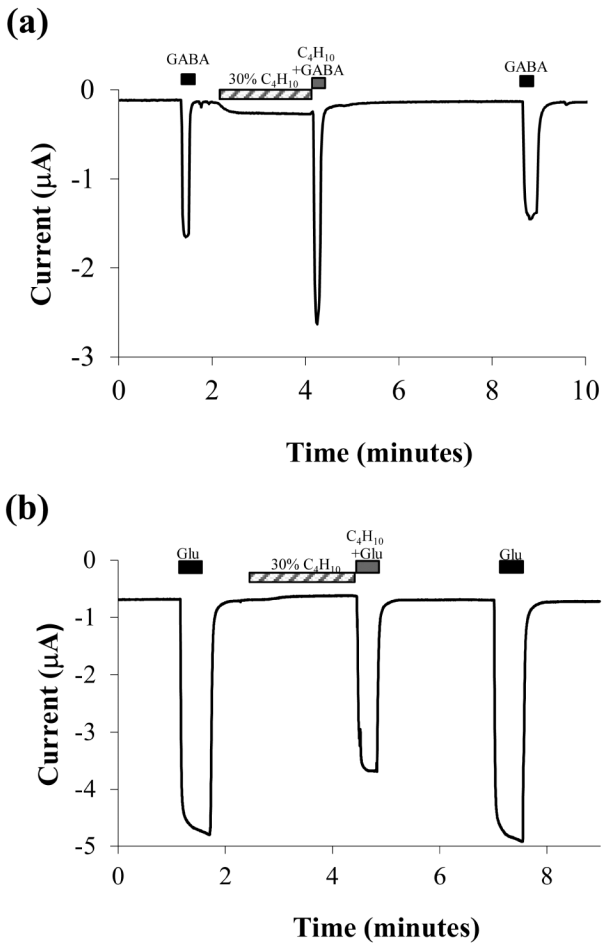

Figure 1.

Sample electrophysiology tracings from two electrode voltage clamp studies of oocytes expressing (a) $GABA_A$ or (b) NMDA receptors before, during, and after exposure to solutions containing approximately 30% of an atmosphere n-butane.

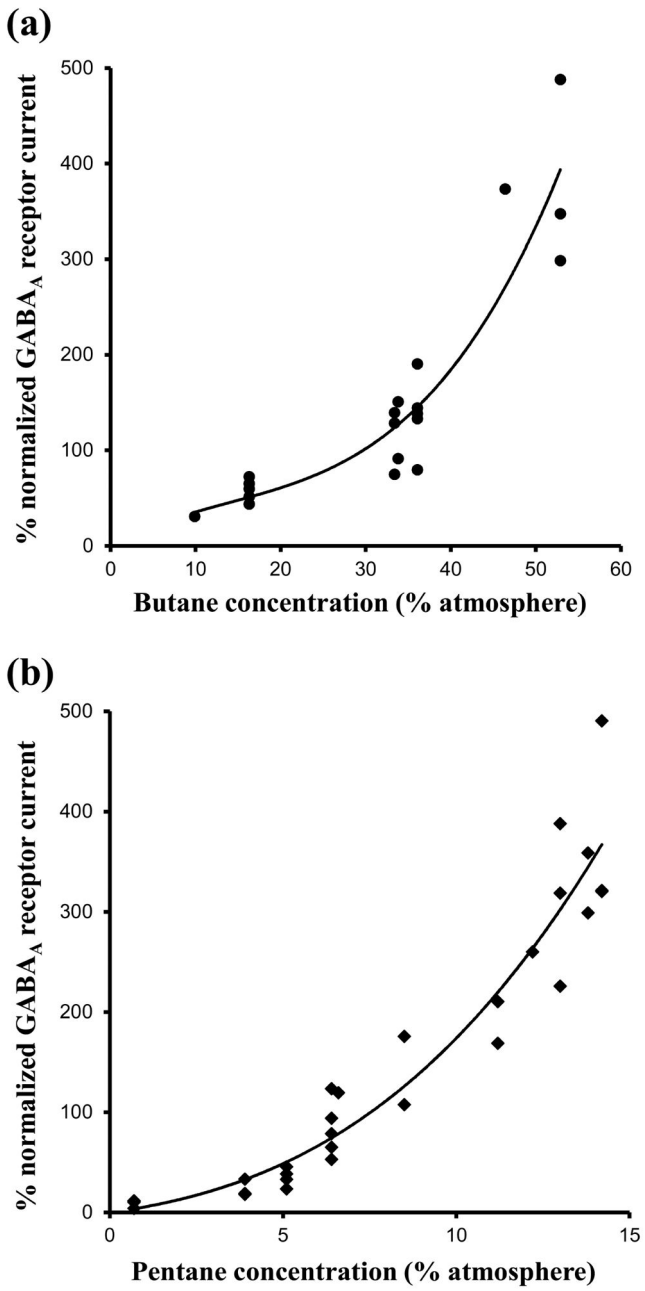

Figure 2.

Concentration-dependent effects of (a) butane or (b) pentane on potentiation of $GABA_A$ receptor currents measured using a two-electrode voltage clamp in oocytes.

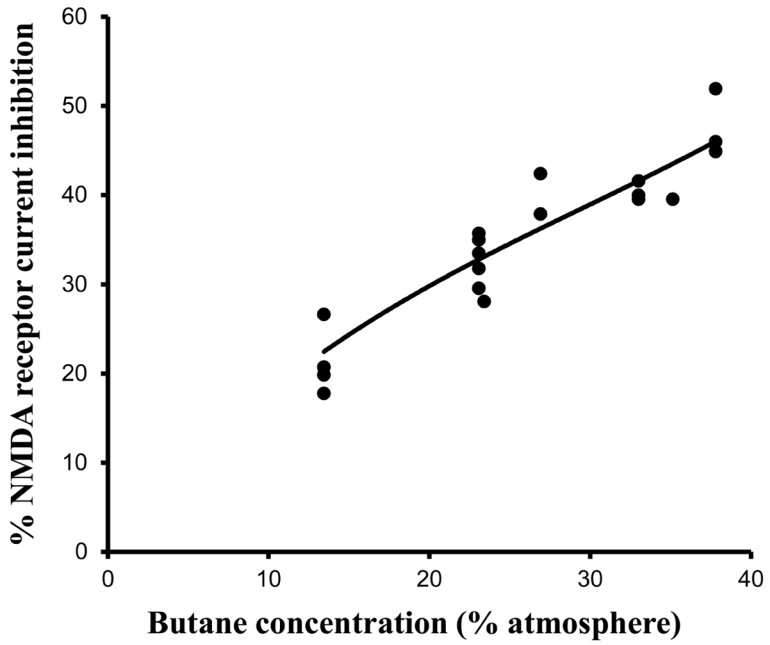

Figure 3.

Concentration-dependent effects of butane on inhibition of NMDA receptor currents measured using a two-electrode voltage clamp in oocytes. Pentane (not shown) did not modulate NMDA receptor currents even at saturated aqueous concentrations.

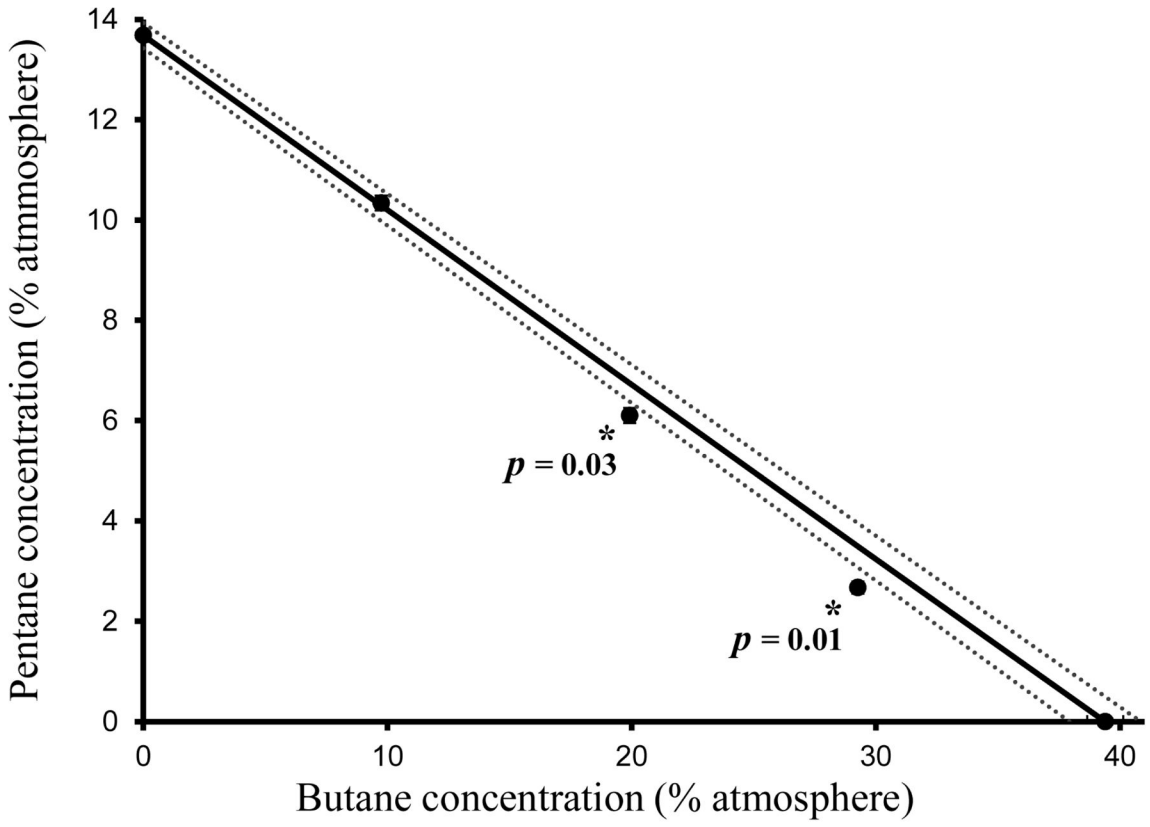

Figure 4.

Isobologram (solid line) with two-tailed 95% confidence bounds (dotted lines) for butane and pentane concentrations at 1 × MAC in rats. Data markers represent the mean values for 8–10 rats each; error bars denote SEM. Combinations significantly outside of the lower isobologram 95% confidence bound are indicated by an asterisk (*) and *p*-value.

Table 1

Sample size, demographic, physiologic, and pharmacologic data for Sprague-Dawley rats anesthetized with butane and/or pentane, expressed as mean ± standard error of the mean, at a total anesthetic dose equal to 1.0 × the minimum alveolar concentration (MAC). The MAC ratio is the target proportion of butane (B) and pentane (P), at MAC, assuming additivity. When administered as single agents, the butane and pentane concentrations equal the butane MAC and pentane MAC, respectively. For n-alkane mixtures, the pentane concentration at MAC is measured during administration of a butane concentration that is held constant throughout the experiment. HR, heart rate; f_R, respiratory rate; SpO_2, hemoglobin oxygen saturation (pulse oximetry); T, temperature.

MAC ratio (B:P)	*n*	Weight (g)	Age (days)	HR (beats minute^{-1})	f_R (breaths minute^{-1})	SpO_2 (%)	T (°C)	Butane (%)	Pentane (%)
1.00 : 0.00	8	291 ± 6	72 ± 1	509 ± 13	133 ± 11	96.3 ± 0.7	37.5 ± 0.1	39.39 ± 0.74	0
0.75 : 0.25	8	292 ± 5	76 ± 1	447 ± 16	155 ± 13	94.0 ± 0.5	37.4 ± 0.1	29.27 ± 0.20	2.67 ± 0.12
0.50 : 0.50	9	284 ± 4	67 ± 1	438 ± 17	135 ± 11	94.8 ± 0.4	37.5 ± 0.1	19.92 ± 0.12	6.10 ± 0.15
0.25 : 0.75	10	305 ± 2	71 ± 0	428 ± 13	121 ± 6	95.3 ± 0.7	37.6 ± 0.0	9.75 ± 0.03	10.34 ± 0.15
0.00 : 1.00	8	282 ± 6	76 ± 2	434 ± 11	121 ± 9	95.7 ± 0.7	37.5 ± 0.1	0	13.68 ± 0.13