Laboratory Investigations

Nonlinear additivity of nitrous oxide and isoflurane potencies in rats

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Purpose: To test the hypothesis that the MAC values of nitrous oxide (N_2O) and isoflurane were not linearly additive, as theorized by the postulated mode of action based on lipid solubility, in a rat model.

Methods: Eight Long Evans rats were randomly assigned to order of measurement of MAC for isoflurane and N_2O alone and in combination using standard 45 sec supramaximal electrical stimulation (50 volts $\times$ 10 msec duration pulses at 50-sec⁻¹ applied for 45 sec sc to the lower abdominal groin area). The MAC of N_2O was measured at hyperbaric compression to 2.25 atmospheres absolute, 1710 mmHg.

Results: The MAC values found were: isoflurane - 0.98 ± 0.12 and N_2O - 1.59 ± 0.12 volume (vol) %, or 1.59 ± 0.12 atmospheres absolute (ATA) (All values are mean $\pm$ standard deviation). The linear additivity theory suggests % MAC agent A + % MAC agent B = 1.0. However, % MAC isoflurane + % MAC N_2O = 1.37 ± 0.15 ($P < .001$).

Conclusion: Nonlinear additivity was demonstrated with direct MAC measurement for both isoflurane and N_2O in rats. This suggests an agonist-antagonist relationship.

Objectif : Évaluer l'hypothèse que les valeurs de CAM (concentration alvéolaire minimale) du protoxyde d'azote (N_2O) et de l'isoflurane ne sont pas additives de façon linéaire, tel qu'envisagé à partir du mode d'action retenu, basé sur la solubilité lipidique, et ce chez le rat comme modèle.

Méthodes : Huit rats de souche Long Evans ont été attribués de façon aléatoire à la séquence de mesure de la CAM pour l'isoflurane et le N_2O de façon isolée ainsi que pour la combinaison des deux. Le test utilisé a été une stimulation électrique supra maximale standard d'une durée de 45 sec (stimuli de 50 volts, d'une durée de 10 msec, d'une fréquence de 50-sec⁻¹ appliqués durant 45 sec par voie sc à la région inguinale basse). La CAM du N_2O a été mesurée en condition hyperbare à 2.25 atmosphères, soit 1710 mmHg.

Résultats : Les CAM trouvées étaient les suivantes: isoflurane: 0.98 ± 0.12 et N_2O : 1.59 ± 0.12 vol % ou 1.59 ± 0.12 atmosphères en valeur absolue (ATA) (toutes les valeurs représentent la moyenne $\pm$ écart type). La théorie de l'addition linéaire propose que % CAM agent A + % CAM agent B = 1. Cependant, dans cette étude, le % CAM isoflurane + % CAM N_2O = 1.37 ± 0.15 ($P < 0.001$).

Conclusion : Une additivité non linéaire a été démontrée par des mesures directes de la CAM de l'isoflurane et du N_2O chez le rat. Ceci suggère une relation agoniste-antagoniste.

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THE mode of action of halogenated anaesthetic agents is theorized to be based upon their lipid solubility. With this theory there is a predicted linear additivity of potencies for different agents.¹ This assessment of gaseous anaesthetic agent potency is reflected by its MAC value, with MAC being the minimum alveolar concentration of a specific agent which prevents purposeful movement in response to supramaximal stimulation in 50% of subjects, whether animal or human.^{2,3} Nonlinear additivity of the potencies of the anaesthetic, but non-halogenated, gas nitrous oxide (N₂O) and the halogenated agents, such as halothane, enflurane and isoflurane has been reported.^{4,5} However, in previous studies, the MAC of the halogenated agent alone has been documented. The N₂O MAC has been extrapolated, but not measured, because determination of N₂O MAC, which is greater than one atmosphere absolute, requires testing under hyperbaric conditions to attain adequate N₂O partial pressures without subject hypoxaemia. If N₂O and halogenated agent potencies are truly non-additive, then different neurophysiological effects and possibly different mechanisms of action can be postulated. We hypothesized that, in a rat model, N₂O and isoflurane would make nonlinear contributions to combined anaesthetic potency. Hyperbaric compression would be utilized during N₂O MAC determinations.

Methods

After approval of the Pennsylvania State University School of Medicine Animal Utilization Committee and in accordance with guidelines on animal care by the United States Department of Health and Human Services, eight Long Evans rats were studied. These were supplied by Charles River [stock designation CrI:(LE)BR] with a pathogen-state classification of Caesarean origin, barrier sustained [COBS], and selected for similar age (approximately 10 wk) and sex (male). All rats were housed in 225 square inch cages, fed the same unrestricted diet of Purina Rat Chow, and had free access to water up to the morning of testing. All studies were begun between 8 and 10 a.m. The rats weighed 417 ± 68 (mean $\pm$ standard deviation) g.

Anaesthesia was induced during spontaneous breathing in a two litre plexiglass box with a total gas flow of 2 l·min⁻¹ oxygen with isoflurane 4.0%. The trachea of each rat was intubated and the lungs mechanically ventilated with a small animal volume ventilator (Harvard Apparatus, Cambridge, MA) to mild hypocapnia with P_{ET}CO₂ of 28–32 mmHg. The ECG was recorded and respiratory gases (inspired oxygen, P_{ET}CO₂, %_{ET}N₂O and %_{ET}isoflurane) were monitored by a calibrated mass spectrometer (MGA 1100, Marquette Gas Monitoring

Co., St Louis, MO).⁶ Each animal was placed on a heating blanket for maintenance of body temperature stability. Temperature did not vary by more than one degree during the experiment.

The MAC values were determined using a standard supramaximal electrical stimulation (50 volts $\times$ 10 msec duration pulses at a frequency of 50 Hz applied for 45 sec to the lower abdominal groin area by a Grass SD-5 Stimulator).^{2,3} The stimulus used was defined as supramaximal because electrical stimulation of higher voltage or longer duration has not been shown to result in a different response from tested animals.³ Stimulations were begun with rat end-tidal anaesthetic concentrations at higher than expected MAC levels and decreased incrementally by approximately 10% after negative responses for three periods of stimulation. A positive response was taken as repetitive, purposeful movement. This did not include tail-wagging activity, back arching, or maintained limb extension. The positive responses to electrical stimulation were elicited more than once in all animals. A minimum 20 min equilibration period was allowed between each decrease and the next stimulus. The MAC values were taken as the concentrations of isoflurane, N₂O or the combination of agents midway between those at which the rat moved purposefully and the next higher at which no response was elicited. During the study, P_{ET}CO₂ was maintained > 25 mmHg, and although during hyperbaric compression it at times exceeded the baseline upper limit of 32 mmHg, did not exceed 45 mmHg in any animal. Normoxic gas mixtures were delivered to all animals throughout the study. No difficulties were encountered with the stimulating electrodes. Tissue burns were not encountered.

The MAC determinations for isoflurane alone were begun at 2% end-tidal isoflurane. The MAC equivalent of combined isoflurane and N₂O was measured at normal atmospheric pressure. Nitrous oxide 70% was administered and isoflurane 1.5% was added. The N₂O dose was chosen because it was approximately 0.5 MAC in rats. The isoflurane was incrementally decreased as described. For the N₂O MAC measurements, isoflurane was discontinued from the ventilating gas and flushed from the respiratory circuit. After end-tidal isoflurane approached 0 (maximum detected end-tidal isoflurane was 0.03%), each animal was compressed to 1710 mmHg, or 2.25 ATA, in an animal hyperbaric chamber (Reimers Engineering, Alexandria, VA). Ventilation was maintained with 1.8 ATA N₂O (equivalent to 180 volume %) and 0.45 ATA oxygen. After 30 min stabilization, testing was begun. The animals were constantly observed by an investigator through a chamber porthole. A period of at least 30 min followed prior to testing.

The values for the combined anaesthetic group (reported in MAC equivalents) and the actual measured MAC values were compared with a one sample *t* test with the expected mean of 1.0. A $P \leq 0.05$ was considered significant.

Results

The MAC of isoflurane was 0.98 ± 0.12 vol % (mean $\pm$ standard deviation). The MAC of N_2O was 159 ± 12 vol %, or 1.59 ± 0.12 atmospheres absolute (ATA). According to the linear additivity theory, when a combination of anaesthetic gases is administered for general anaesthesia, the % MAC of one agent added to the % MAC of a second agent should be additive to a % MAC equivalent of 1.0 MAC. When administered simultaneously, MAC measurements showed that the % MAC isoflurane + % MAC N_2O was 1.37 ± 0.15 MAC equivalents ($P < 0.001$). Approximately 37% more anaesthetic agent was required in combination than when alone to produce the same effect.

Discussion

In Long-Evans rats, there is nonlinear additivity for the potencies of the halogenated anaesthetic agent isoflurane and the anaesthetic gas N_2O when administered simultaneously. The individual MAC values for isoflurane, although different from that reported for Sprague-Dawley rats,⁷ and N_2O have been measured and reported for other rats of the same strain and found to be no different.^{7,8}

The unitary theory of narcosis assumed a single mechanism of anaesthetic action upon the cell lipid membrane with linear additivity of each agent's potency.¹ The multisite theory of anaesthetic action questioned this assumption of anaesthetics causing a single cell membrane effect.⁹ The analgesic properties of N_2O has been suggested to be secondary to effects at opiate receptors.¹⁰ However, demonstrations that naloxone has no effect on N_2O anaesthesia have refuted this as a full explanation for its activity.¹¹ Recent theories of anaesthetic action have also postulated that there is anaesthetic agent binding to specific membrane proteins (perhaps agent-specific proteins to some degree) rather than, or in addition to, disruption of cell membrane lipids.¹² The findings of nonlinear additivity of anaesthetic agent potency has also contributed to the idea that there may not be only one mechanism or site of action.^{4,5}

The MAC value for N_2O in this study was the same as the value previously reported by Russell and Graybeal^{7,8} and similar to the 147 ± 7.6 vol % (1.47 ± 0.076 ATA) reported for N_2O MAC by Wardley-Smith and Halsey⁹. Measured MAC for N_2O differs

somewhat from the value of 136 vol % (1.36 ATA) predicted from its lipid solubility and that postulated from MAC determinations of N_2O and halothane together when halothane MAC had previously been directly measured.¹³ It is less than the 220 vol % (2.20 ATA) value assumed if enflurane and N_2O potencies were linearly additive.⁵ The MAC of isoflurane measured in this study is the same as the 0.98 ± 0.14 previously determined by Russell and Graybeal using the same technique.⁷

The concept of additive MAC was originally introduced when it was noted that 70% N_2O caused a 61% reduction in halothane anaesthetic requirement.¹⁴ This has since been shown to be the case for many combinations of anaesthetic agents. DiFazio *et al.*, showed that cyclopropane added to teflurane or halothane exhibited additivity, but not when added to N_2O or ethylene.¹³ The idea of non-additivity was raised early in the development of the MAC concept. Cullen *et al.*, showed additivity of xenon and halothane, with xenon having a MAC of 71% in man.¹⁵ Miller *et al.*, also demonstrated additivity of ethylene and halothane without synergism or antagonism.¹⁶

However, the concept of the agonist-antagonist relationship between some anaesthetic agents has persisted. Wardley-Smith and Halsey showed that different anaesthetics did not necessarily have additive potencies when used as mixtures.⁹ Although studying mixtures of different intravenous agents with each other and N_2O may not be the same as mixtures of two gaseous agents, the effects were demonstrated. Althesin and N_2O and methohexitone and N_2O were not additive, whereas thiopentone and N_2O and thiopentone and propofidid were.

Cole *et al.*, demonstrated a dose-dependent interaction between N_2O (0–80%) and enflurane.⁴ Increasing N_2O from 0–10%, from 30–60% or 70–80% did not decrease enflurane requirements, but increasing N_2O from 10–30% or 60–70% did. Synergism at low levels or antagonism at higher concentrations were postulated to occur. Cole *et al.*, also demonstrated non-linear potency effects of N_2O in rats when administered with enflurane, halothane, and isoflurane with N_2O administered in sub-MAC doses of up to 75%, although N_2O MAC was extrapolated in the rats, not measured.^{4,5}

There are specific concerns about factors that may influence results when performing MAC studies. The measured MAC values of an anaesthetic can be altered by differences in animal physiology. For example, MAC decreases with age,^{17–19} but in the present study all animals tested were of similar age and weight. Temperature was maintained and stable throughout the experiments and $P_{ET}CO_2$ did not exceed 45 mmHg in any animal. Higher carbon dioxide levels are required to affect

anaesthetic susceptibility. The initial isoflurane MAC measured and the final N₂O MAC values are in keeping with values previously recorded with the same methodology. The combined MAC values were done between these two measurements, so physiological perturbations were not a factor in results obtained.

The noxious stimulus used for MAC determination was electrical stimulation rather than clamping of the proximal tail, which has been used in many MAC studies.^{3-5,20} Determination of MAC by supramaximal electrical stimulation has been well documented to be a reliable and accurate noxious stimulus for MAC determination in rats,^{1,2,19} mice,²¹ dogs,^{22,23} and humans.¹⁴ The electrical stimulation was applied subdermally by a needle electrode to the lower abdomen in the groin area. This location was felt to decrease the likelihood of the needle electrode becoming disconnected from the animal during movement associated with positive responses while still inside the closed hyperbaric chamber. The likelihood of tissue burns was also felt to be reduced. In previous MAC studies electrical stimulation has been applied to the base of the tail, the cheek and other membrane surfaces.² Ten volt electrical stimuli have been reported to be painful and useful for MAC measurement when applied by needle electrodes to the human forearm.^{2,14} Electrical stimulation has also been reliably used with stimulating electrodes placed *sc* in the lower abdomen (as in the present study) and the perineum.²² Inside an animal hyperbaric chamber, remotely controlled tail clamp applications were found to be technically unreliable. Remote control of the electrical stimulation functioned consistently. Although tail clamps could have been applied during normobaric testing the same noxious stimulus was required for all.

The present study must acknowledge several limitations. Electrical stimulation, instead of tail clamping, was used as the noxious stimulus. However, studies have previously documented the reliability and accuracy of this methodology. The potential for tolerance to develop for N₂O does exist. The question of anaesthetic tolerance, which could possibly have affected MAC measurements, was not addressed by this study. Tolerance development to N₂O, but not to the halogenated anaesthetics, such as isoflurane, has been postulated to occur in both animals and man. Tolerance has been shown to be present in mice, but it is suggested to occur after the first few minutes of anaesthetic exposure.^{24,25} However, tolerance could not be demonstrated in present studies. We cannot be certain that isoflurane was washed out of tissues. The MAC values for each agent and for the combined agents were determined in each animal. Therefore, although possible, since the final N₂O MAC was the same as that found in

other animals exposed to isoflurane for instrumentation only early in a study, it is very unlikely that results were influenced. All animals were ventilated equally, end-tidal respiratory gases were measured by mass spectrometry. Time intervals between noxious stimuli and MAC determinations were similar to those used in previously published studies.

The MAC of N₂O was measured in a hyperbaric chamber at 2.25 ATA. Pressure reversal of anaesthetic effects occur, but it has been demonstrated at hyperbaric pressures far greater, often in excess of 100 ATA, than those used in this study.^{26,27} The initial MAC measurements for isoflurane and the final N₂O MAC measurements were the same as those previously recorded in this laboratory.^{7,8} In spite of these possible limitations, the present results demonstrated that there is a non-linear additivity of anaesthetic potency for the halogenated anaesthetic isoflurane and N₂O.

For the first time, nonlinear additivity was demonstrated with direct MAC measurement for both the halogenated agent and N₂O. With regards to anaesthetic potency, this relationship suggests an agonist-antagonist relationship between isoflurane and N₂O and supports the suggestion of a different mechanism of action, without contributing to its delineation.

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