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Selective Breeding Alters Murine Resistance to Nitrous Oxide Without Alteration in Synaptic Membrane Lipid Composition

Donald D. Koblin, Ph.D., David E. Dong, A.B.,† Joan E. Deady, M.S.,† Edmond I. Eger, II, M.D.‡*

A normal population of mice was separated into two groups with reproducibly high (>1.63 atm) or reproducibly low (<1.29 atm) nitrous oxide requirements. Males (n = 4) and females (n = 5) with the reproducibly high anesthetic requirements were mated, as were males (n = 4) and females (n = 3) with the reproducibly low anesthetic requirements. The first-generation offspring from parents with the high anesthetic requirements had a higher nitrous oxide ED_{50} (concentration of nitrous oxide required to abolish the righting reflex in half of the animals) than did offspring from parents with the low anesthetic requirements. Mice with the lowest and the highest anesthetic requirements in the first generation were bred to give the second generation. By repeating this process of breeding, nitrous oxide ED_{50} testing, and selection of mice with the highest and lowest anesthetic requirements through five generations, the authors were able to breed two groups of mice separated by approximately 0.5 atm in nitrous oxide requirements. This alteration in anesthetic requirement could not be explained by an altered synaptic membrane lipid composition, since no significant difference in synaptic membrane phospholipid, fatty acid, or cholesterol compositions could be detected in the two groups of mice with high and low anesthetic requirements. (Key words: Anesthetics, gases: nitrous oxide. Brain: lipids, synapses. Genetic factors. Potency: ED_{50}; righting reflex. Theories of anesthesia: lipid solubility.)

ANESTHETIC REQUIREMENTS vary slightly among animals of a given species. However, the variability for a given animal is less than the variability of the population from which that animal was drawn.[1] The reproducible nature of the anesthetic requirement for a single animal suggests that the different values among animals do not simply represent day-by-day variation of a homogeneous population. This implies a distribution of anesthetic requirements that may allow a normal population to be divided into resistant and vulnerable members. The present report documents this thesis for nitrous oxide requirements in mice. In addition, we show that these differences in anesthetic requirements may be transferred to the offspring.

Because of the near-perfect correlation between lipid solubility and anesthetic potency,[2,3] and because of the ability of general anesthetics to perturb the physical state of phospholipid model membranes,[4–8] we postulated that mice resistant to and susceptible to nitrous oxide anesthesia might differ in their neuronal membrane lipid compositions. We therefore examined the synaptic membrane fatty acid, phospholipid, and cholesterol compositions in the mice with high versus low anesthetic requirements.

Methods and Materials

All mice employed in these experiments were fed Purina® laboratory chow and water *ad libitum*, and were maintained on a 12 hr:12 hr light:dark cycle.

The righting reflex was examined in 500 male and 100 female stock CD-1 mice (Charles River), weighing 25 to 30 g, at N_2O partial pressures of 1.29, 1.46, and 1.63 atm. Eight unrestrained mice were placed in individual wire mesh cages that could be rotated at 4 rpm in a 20-liter hyperbaric chamber.[9] Chamber temperature was adjusted with circulating water heat exchangers to maintain rectal temperatures of two additional restrained mice between 36.5 and 38.0 C. Circulation of chamber gases through a soda lime container removed carbon dioxide. The hyperbaric chamber was flushed with 100 per cent oxygen for 10 min before 1.29 atm N_2O was added. After a 30-min equilibrium, animals rolling over twice during five complete turns of the rotator failed the test and were considered anesthetized. Two further additions of N_2O were made in increments of 0.17 atm, and animals were re-examined at each dose, following a 15-min equilibration, for their ability to right themselves.

Mice that failed the righting reflex test at 1.29 atm or more were placed into a LO group. Those that passed this test at 1.63 atm or below were placed in a HI group. Each LO and HI animal was then retested three or four more times at weekly intervals. Only mice that consistently failed the righting reflex test at 1.29 atm N_2O were kept in the LO group, and only mice that consistently passed the righting reflex test at 1.63 atm N_2O were kept in the HI group. We obtained four males and three females in the LO group and four males and five females in the HI group.

LO male mice were mated with LO female mice, and HI males with HI females. The N_2O ED_{50} (the partial pressure of N_2O required to abolish the right-

* Assistant Research Chemist.
† Staff Research Associate.
‡ Professor of Anesthesiology.

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Address reprint requests to Dr. Koblin.

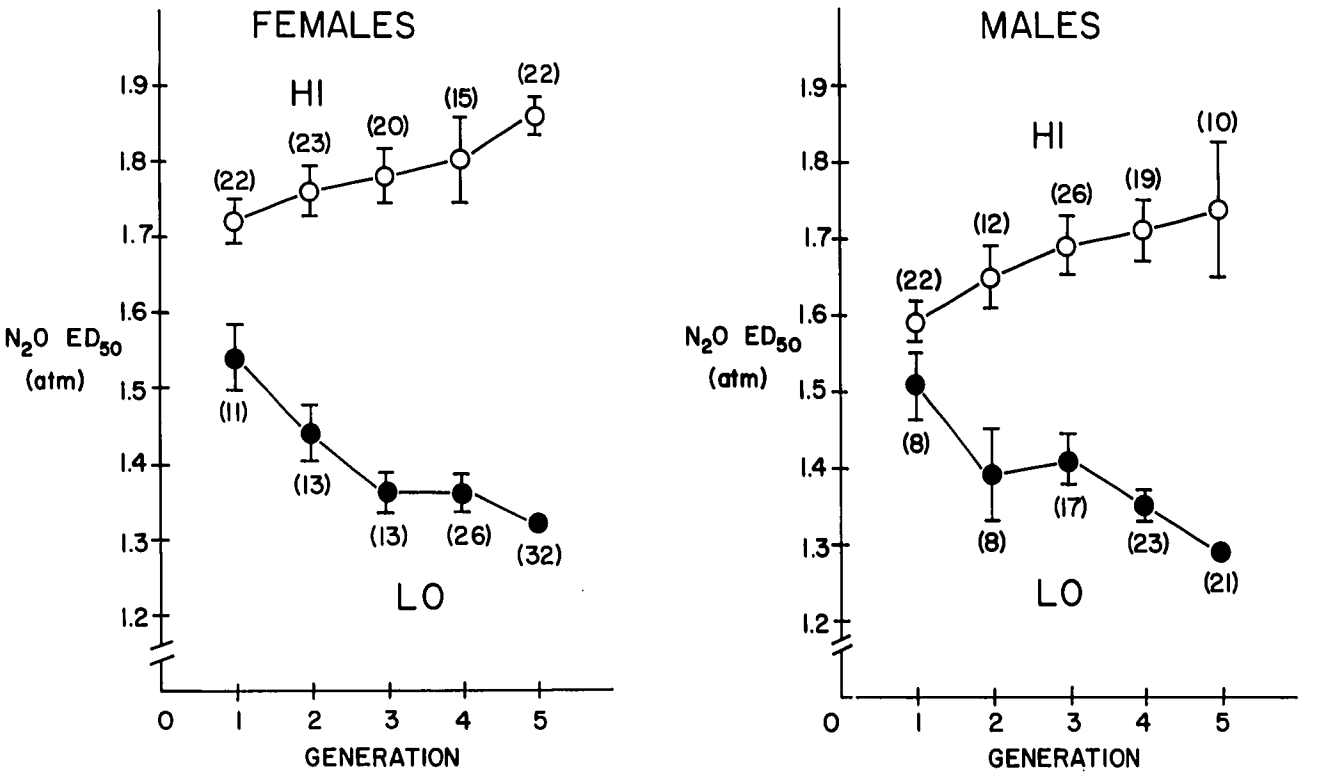

FIG. 1. Nitrous oxide ED_{50} values ± SE in the offspring of mice that were selectively bred for their resistance to (HI group) and susceptibility to (LO group) nitrous oxide anesthesia. Number in parentheses indicates the number of mice tested for the individual ED_{50} value. The differences in nitrous oxide requirements for the first-generation HI and LO females were statistically significant ($P < 0.001$).

ing reflex in half the animals) was determined under controlled-temperature conditions in the offspring of these mice (first generation). Mice were placed in the hyperbaric chamber, the chamber was flushed with O_2 for 10 min, and 1.22 atm N_2O was added. Following a 30-min equilibration, animals were examined for their ability to pass the righting reflex test. Nitrous oxide was added or removed in 0.11-atm steps, and the mice were retested after a 15-min equilibration. Oxygen always remained above 0.6 atm during testing. Concentrations of N_2O were determined by gas chromatography. Anesthetic requirement was calculated for each individual by averaging the nitrous oxide partial pressures (concentration times chamber pressure) that just permitted and prevented the animal from righting itself. The ED_{50} and standard error values for a group of mice were calculated from these individual crossover values. If an animal died during the study, its responses were not included in the ED_{50} calculations. Significance was calculated by use of a t test for unpaired data. All ED_{50} measurements were performed with the observer unaware of the identity of the animals.

The males and females with the lowest and the highest anesthetic requirements in the first generation were bred to give the second generation. This process of breeding, N_2O ED_{50} testing, and selection of mice with the lowest and highest anesthetic requirements was repeated through five generations. For breeding, one male was mated with one to three females. We avoided brother–sister matings. Mice were weaned at three weeks of age, and N_2O ED_{50} measurements were performed at two to four months of age.

Synaptic plasma membranes were prepared from whole brains of the HI and LO mice after the procedure of Jones and Matus.[10] Brains were homogenized in 10 per cent (w/w) sucrose and the homogenate centrifuged at 800 *g* for 20 min to remove the nuclear pellet. The supernatant was centrifuged at 9,000 *g* for 20 min to obtain the crude mitochondrial pellet, which was washed once in 10 per cent (w/w) sucrose. The crude mitochondrial pellet was lysed in 5 mM tris (*p*H 8.1 at 4 C), and the suspension was mixed with a 48 per cent (w/w) sucrose solution to give a final concentration of 34 per cent (w/w) sucrose. The sample suspension was overlaid with a 28.5 per cent (w/w) and a 10 per cent (w/w) sucrose solution, and was centrifuged for 110 min at 21,500 rpm on

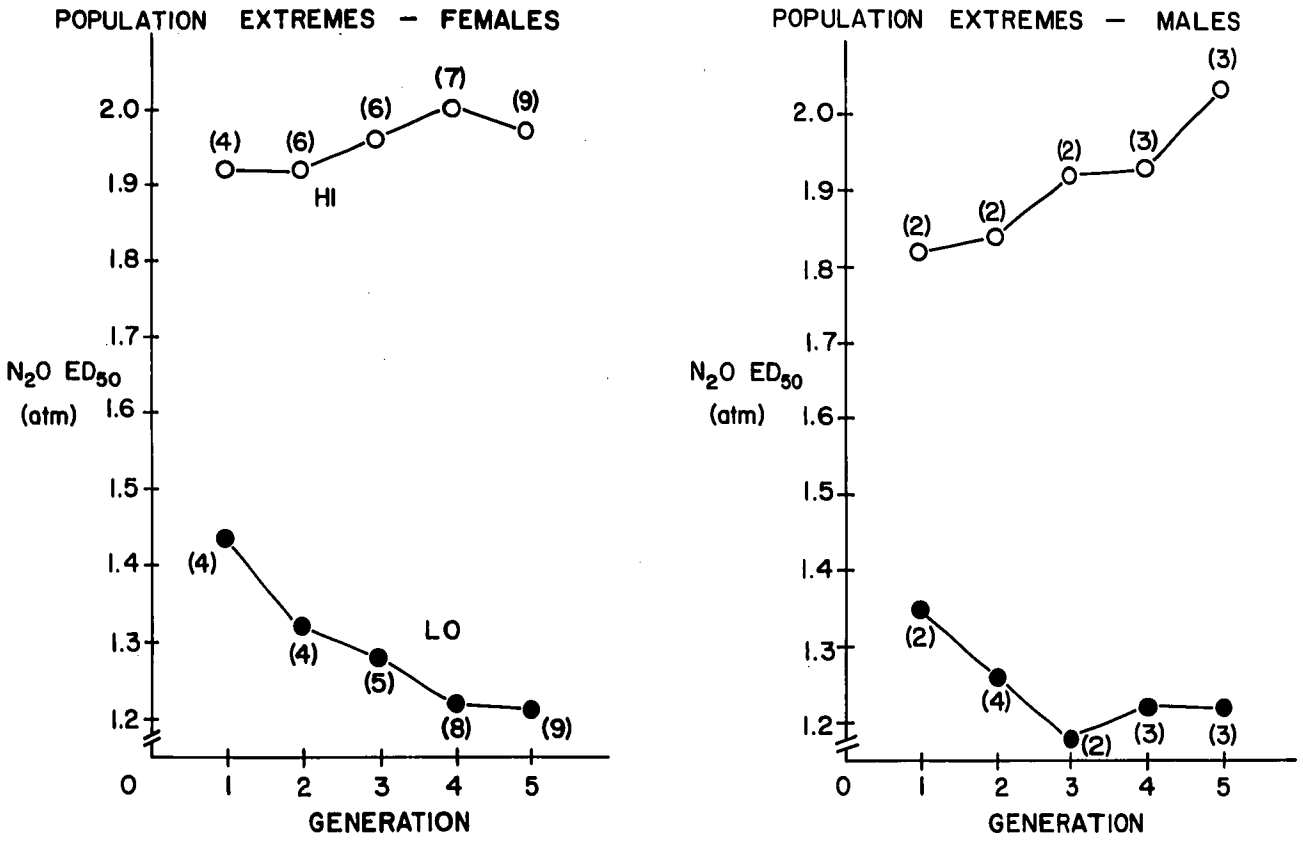

FIG. 2. Selection for breeding of mice with the lowest nitrous oxide requirements in the LO group and the highest nitrous oxide requirements in the HI group, to give the following generation of HI and LO animals. Number in parentheses represents the number of animals selected for mating. Each point represents the average nitrous oxide requirement for the number of animals selected.

a Beckman SW27 rotor. An enriched fraction of synaptic membranes separated at the 28.5–34 per cent sucrose interface, myelin at the 10–28.5 per cent sucrose interface, and mitochondria sedimented to the bottom. Sucrose solutions were buffered with 5 mM HEPES, *p*H 7.4. Procedures were carried out at 0–4 C and all solutions were saturated with argon to minimize lipid oxidation.

We tested for separation of cellular components by various enzymatic assays. $Na^+ + K^+$ ATPase, acetylcholinesterase, lactate dehydrogenase, and succinate dehydrogenase activities were measured in the homogenate, myelin, synaptic plasma membrane, and mitochondrial fractions for each preparation. Synaptic plasma membranes showed a two- to threefold enrichment in $Na^+ + K^+$ ATPase and acetylcholinesterase activities and low levels of lactate dehydrogenase and succinate dehydrogenase activities compared with the homogenate.

Synaptic membrane lipid analyses were performed on selected mice from the fourth and fifth generations that had individual anesthetic requirements lower than that of the LO group average or higher than that of the HI group average. Mice selected from the LO group had an average nitrous oxide requirement of 1.27 ± 0.01 atm (±SE; n = 24, 12 males and 12 females). Mice selected from the HI group had an average nitrous oxide requirement of 1.88 ± 0.03 atm (±SE; n = 21, 7 males and 14 females). Brains from two or three mice of the same sex were pooled for each synaptic membrane preparation. Since no significant effect due to sex was seen in the fatty acid, phospholipid, or cholesterol compositions, the results for the two sexes were combined.

Lipids were extracted from synaptic membranes by sonication with methanol once and with a 1:1 chloroform:methanol solution twice. Synaptic membrane phospholipids and cholesterol were separated by one-dimensional thin-layer chromatography (TLC) with a solvent system consisting of chloroform:methanol:acetic acid:water in the ratios of 65:50:5:3. Phospholipid fatty acids were transesterified to their methyl esters after a modification of the Morrison and Smith[11] procedure, and analyzed on a SP-2330 cyanosilicone

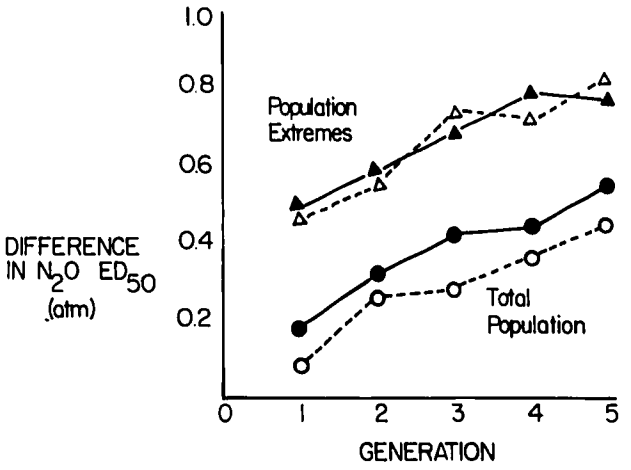

FIG. 3. Separations of HI and LO nitrous oxide ED_{50} values in five successive generations. Circles represent the differences between nitrous oxide ED_{50} values for the total populations of HI and LO animals: O, males; ●, females. Triangles represent the differences in nitrous oxide ED_{50} values for the population extremes of HI and LO animals selected for breeding; Δ, males; ▲, females. The HI and LO population extremes selected from the original stock CD-1 mice (generation zero) were separated by at least 0.34 atm in anesthetic requirements.

column in a Hewlett Packard 5830A gas chromatograph with digital integrator. Fatty acid methyl esters were analyzed over a 180–230 C temperature range, programmed to increase at 2 degress C/min. Phospholipids were quantitated by measuring inorganic phosphate released following perchloric acid digestion.[12] Cholesterol was determined by gas chromatography after reaction with trimethylsilylimidazole. Further details on the lipid analysis procedures are presented elsewhere.[13]

Results

The first-generation offspring of LO-group parents had a lower N_2O ED_{50} than did the first-generation offspring of HI-group parents (fig. 1). First-generation females in the HI and LO groups were separated by 0.18 atm N_2O in anesthetic requirements (fig. 1, *left*) and males by 0.08 atm (fig. 1, *right*). The differences in nitrous oxide requirements for the first-generation HI and LO females were statistically significant. Progressively greater ED_{50} separations were obtained for each generation, for both females (fig. 1, *left*) and males (fig. 1, *right*). Fifth-generation females were separated by 0.54 atm and fifth generation males by 0.45 atm in nitrous oxide requirements.

The average nitrous oxide requirements in the first-generation LO and HI population extremes of mice taken for breeding were separated by approximately 0.5 atm (fig. 2). By the fifth generation, the LO and HI population extremes were separated by about 0.8 atm (fig. 2). The lowest of the LO mice had a crossover value of 1.19 atm N_2O and the highest of the HI mice had a crossover value of 2.07 atm N_2O. The separations of HI–LO nitrous oxide ED_{50} values for the total population and for the population extremes in five successive generations are summarized in figure 3.

We analyzed the fatty-acid compositions of the five major synaptic membrane phospholipid components: sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine (table 1). The fatty-acid compositions of synaptic membrane phospholipids isolated from the HI mice did not differ significantly from those isolated from the LO mice (table 1). In addition, the phospholipid compositions and the cholesterol/phospholipid molar ratios of synaptic membranes isolated from the HI and LO mice were not significantly different (table 2). Whole-brain homogenate and synaptic membrane acetylcholinesterase and $Na^+ + K^+$ ATPase activities were the same in the HI and LO animals (data not presented).

Discussion

These studies demonstrate that a population of mice can be separated into two groups with reproducibly high or low nitrous oxide requirements, and that these differences can be transferred to the offspring. Previous studies have shown selection for phenotypes that are directly related to responses to other drugs.[14] Rats have been selected for high and low alcohol preferences.[15] Mice can be bred to differ in their sensitivities to the hypnotic effect of ethanol.[16] The genetic control of levorphanol-induced locomotor activity has been demonstrated in mice.[17] In addition, recombinant inbred mouse strains, their reciprocal F1 hybrids, and their progenitor strains show strain differences in times to induce and recover from halothane anesthesia.[18]

In our experiments, nitrous oxide has an advantage over most other agents as a genetic selection agent since it is essentially unmetabolized, and equilibration of inspired and brain nitrous oxide partial pressures occurs quickly.[19] This means that the above-described separation of nitrous oxide requirements results from an alteration in the sensitivity of the central nervous system to nitrous oxide rather than from an alteration in metabolism, absorption, distribution, or secretion. However, it remains to be seen whether these HI and LO mice are also resistant and susceptible to other general anesthetics. Furthermore, anesthetic potencies in the HI and LO mice will eventually have to be measured employing an endpoint for anesthesia other than the righting reflex (*e.g.*, response to a painful stimulus), to insure that the separations in anesthetic requirements indicate a general resistance or

TABLE 1. Fatty-acid Compositions of Synaptic Membrane Phospholipids Isolated from Mice Bred for Their Resistance or Susceptibility to Nitrous Oxide Anesthesia*

Fatty Acid†	Sphingomyelin		Phosphatidylcholine		Phosphatidylserine		Phosphatidylinositol		Phosphatidylethanolamine	
	HI‡	LO§	HI	LO	HI	LO	HI	LO	HI	LO
Palmitate 16:0	7.4 ± 0.9¶	9.4 ± 1.1	53.5 ± 0.5	53.7 ± 0.4	1.5 ± 0.1	1.4 ± 0.1	9.3 ± 0.4	10.7 ± 0.7	8.7 ± 0.2	8.5 ± 0.2
Palmitoleate 16:1 (n − 9)	0.3 ± 0.2		0.9 ± 0.0	0.9 ± 0.0				0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.0
Stearate 18:0	87.4 ± 1.8	83.5 ± 2.1	11.8 ± 0.1	11.8 ± 0.1	46.2 ± 0.8	46.0 ± 1.0	43.4 ± 0.9	43.4 ± 1.3	27.7 ± 0.3	27.5 ± 0.6
Oleate 18:1 (n − 9)	2.9 ± 0.9	3.8 ± 0.8	22.9 ± 0.2	23.1 ± 0.2	7.1 ± 0.3	6.3 ± 0.3	5.9 ± 1.0	5.7 ± 0.5	8.1 ± 0.2	7.6 ± 0.2
Linoleate 18:2 (n − 6)			0.5 ± 0.1	0.4 ± 0.0				0.1 ± 0.1		
Linolenate 18:3 (n − 6) and/or Arachidate 20:0	2.1 ± 0.4	2.8 ± 0.2	0.1 ± 0.0					0.1 ± 0.1		
Eicosenoate 20:1			1.1 ± 0.1	1.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0		0.1 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Eicosadienoate 20:2			0.2 ± 0.1	0.1 ± 0.0						
Eicosatrieonate 20:3 (n − 6)			0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Arachidonate 20:4 (n − 6)			3.9 ± 0.1	4.0 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	36.7 ± 1.4	34.4 ± 1.7	11.8 ± 0.1	12.1 ± 1.2
Decosatetraenoate 22:4 (n − 6)			0.7 ± 0.2	0.5 ± 0.0	3.1 ± 0.0	3.0 ± 0.1			4.9 ± 0.1	5.0 ± 0.1
Docosapentaenoate 22:5 (n − 6)					0.3 ± 0.1	0.3 ± 0.1			0.3 ± 0.0	0.3 ± 0.0
Docosapentaenoate 22:5 (n − 3)					0.2 ± 0.0	0.2 ± 0.0			0.4 ± 0.0	0.6 ± 0.3
Docosahexaenoate 22:6 (n − 3)			4.0 ± 0.1	4.1 ± 0.1	39.4 ± 0.7	40.8 ± 0.9	4.5 ± 0.3	4.9 ± 0.4	37.0 ± 0.5	37.9 ± 0.6

* Expressed as weight per cent of the listed fatty acids. Values less than 0.1 per cent are not listed.

† Fatty acids are expressed in the form x:y (n − z), where x is the number of carbon atoms in the fatty acid chain, y is the number of double bonds, and z is the number of carbon atoms from the terminal methyl group end of the fatty acid chain at which the first unsaturation occurs. Thus linoleic acid, 18:2 (n − 6), is 18 carbon atoms long and has two unsaturations occurring between the sixth and seventh and between the ninth and tenth carbon atoms from the terminal methyl group end of the fatty acid chain.

‡ HI mice had an average nitrous oxide requirement of 1.88 ± 0.03 atm (±SE; n = 21).

§ LO mice had an average nitrous oxide requirement of 1.27 ± 0.01 atm (±SE; n = 24).

¶ Mean values ±SE of seven separate synaptic plasma membrane preparations in the HI group and nine separate synaptic plasma membrane preparations in the LO group.

TABLE 2. Phospholipid Compositions and Cholesterol: Phospholipid Molar Ratios of Synaptic Plasma Membranes Isolated from Mice Bred for Their Resistance or Susceptibility to Nitrous Oxide Anesthesia

	LO	HI
Sphingomyelin* (SPH)	3.8 ± 0.3†	4.4 ± 0.2
Phosphatidylcholine (PC)	41.1 ± 0.8	39.5 ± 0.5
Phosphatidylserine (PS)	14.0 ± 0.6	15.3 ± 1.1
Phosphatidylinositol (PI)	2.8 ± 0.2	3.0 ± 0.2
Phosphatidylethanolamine (PE)	38.2 ± 1.0	37.7 ± 0.8
Molar ratio‡ cholesterol/phospholipid	0.591 ± 0.017	0.582 ± 0.019

* Phospholipid content is calculated as the per cent phosphate composition.

† Mean ± SE from seven separate synaptic plasma membrane preparations in the HI group and nine separate synaptic plasma membrane preparations in the LO group.

‡ Calculated from the amount of cholesterol recovered and the amount of phosphate recovered in the SPH, PC, PS, PI, and PE fractions of the TLC plate.

susceptibility to anesthesia rather than a specific selection of mice with different righting response capabilities.

The only notable difference in physical appearances between the HI and LO mice was that about 5 per cent of the HI mice in the fourth and fifth generations showed extensive hair loss. However, these "hairless" mice showed no detectable difference in anesthetic requirements compared with the rest of the HI-group animals. No obvious behavioral difference between the HI and LO mice was seen. No weight difference between the two groups was observed.

The excellent correlation between anesthetic potency and lipid solubility[2,3] and the ability of general anesthetics to fluidize phospholipid[4-8] and alter the physical state of biological membranes[4,20] suggested to us that our approach might have selected mice with altered neuronal membrane lipid compositions. For instance, the HI mice might resist the fluidizing effects of anesthetics by having a higher content of saturated fatty acids or cholesterol or a larger amount of phospholipids with higher phase transition temperatures. However, fatty-acid, phospholipid, or cholesterol compositions did not differ between the two groups of mice separated by 0.61 atm in nitrous oxide requirements. Although we found no significant difference in synaptic membrane fatty-acid, phospholipid, or cholesterol compositions in the HI and LO animals, synaptic membrane lipids may still play a role in explaining the alterations in nitrous oxide requirements. Gangliosides constitute about 10 per cent of the synaptic plasma membrane lipids[21] and were not analyzed in this study. In addition, synaptic membrane lipids contain a significant proportion of alkenyl acyl *sn*-glycero-3 phosphoryl ethanolamine,[22,23] but for the present investigation the alkenyl acyl compounds were not examined separately from the diacyl compounds, and the alkenyl derivatives were not quantitated. Furthermore, if the alterations in anesthetic requirement were the result of a specific change in fatty-acid, phospholipid, or cholesterol composition of synaptic membranes in one small brain region, or the result of an alteration in only a small region of the synaptic membranes (such as the region neighboring membrane proteins), the present techniques would not be sensitive enough to detect such a change. We are presently searching for other explanations, since demonstration of the structural change that produces the difference in anesthetic requirements may indicate the answer to a broader question: how do anesthetics act?

The present experiments may also have clinical implications. The extremes achieved by breeding imply that variations in patient responses may represent basic differences in resistance to anesthesia. The difference in the occasional patient who is unusually susceptible or resistant to anesthesia may not be the result of a pharmacologic or physiologic effect such as drug abuse or change in body temperature alone. Instead, tolerance or intolerance to anesthesia may result from genetically defined differences in the central nervous system.

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