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Mechanisms of General Anesthesia:

Failure of Pentobarbital and Halothane to Depolymerize Microtubules in Mouse Optic Nerve

Albert J. Saubermann, M.D.,* and Martha L. Gallagher, B.S.†

Optic nerves of mice given 40 mg/kg sodium pentobarbital, 1.5 per cent halothane, or no anesthetic were examined by electron microscopy. Microtubule and microfilament densities were analyzed to determine whether general anesthesia occurs with or without depolymerization of axonal microtubules *in vivo*. Microtubule density was inversely related to axonal cross-sectional area. No quantitative difference between either experimental group and control was observed. Narcosis can be produced without depolymerization of axonal microtubules in mouse optic nerve. (Key words: Microtubules; Pentobarbital; Halothane; Theories of anesthesia.)

MICROTUBULES are ubiquitous structures found in many different cells. Under certain conditions microtubules disaggregate or depolymerize, thus morphologically changing from a tubular and easily recognized form to their protein subunits, which appear amorphous when examined by electron microscopy. Often when these conditions are reversed microtubules can be seen to reappear with their original morphology. Allison and Nunn [1] proposed the hypothesis that general anesthetics produce narcosis by reversible depolymerization of microtubules in nerve cells, and they cite circumstantial evidence in support of this hypothesis. Allison *et al.*[2] were also able to demonstrate that disappearance of microtubules in *Actinosphaerium* axopods was caused by clinical concentrations of many common anesthetics; on removal of the anesthetics, the microtubules returned. We have examined the microtubule populations in the optic nerves of mice under deep pentobarbital and deep halothane anesthesia and have been unable to demonstrate microtubule disappearance.

* Research Fellow and Resident in Anaesthesia, Harvard Medical School and Beth Israel Hospital.
† Research Assistant, Department of Anaesthesia, Harvard Medical School and Beth Israel Hospital.

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Methods

Nine 42-day-old male CD-1 strain Swiss albino mice (Charles River Breeding Labs) were divided into three groups. The first three mice received 40 mg/kg sodium pentobarbital (Diabutal, Diamond) in saline solution (concentration 10 mg/ml) intravenously, given as rapidly as possible (less than 2 sec) through a tail vein. Another three mice were given halothane at an inspired concentration of approximately 1.5 per cent in a specially constructed anesthesia chamber[3] of known volume containing CO_2 absorbent and O_2 supplied from two 5-ml reservoir syringes. The third group of three mice served as controls. All animals were sacrificed by decapitation. The calvarium was removed and the brain lifted so that no tension was put on the optic nerves. The optic nerves could thus be fixed *in situ* with 5 per cent glutaraldehyde in cacodylate buffer at 20 C, *p*H 7.4, before being removed and further fixed in additional cacodylate-buffered 5 per cent glutaraldehyde at 20 C. Time from decapitation to fixation averaged 75 sec. The tissue was postfixed in Dalton's chrome osmium, stained *en bloc* in uranyl acetate, dehydrated in acetone, embedded in Epon-Araldite and examined with a Philips 200 electron microscope. Each optic nerve of each mouse was divided into three parts just distal to the optic chiasm and oriented along the longitudinal plane during embedding. Mice given pentobarbital were sacrificed 2 minutes after injection; mice given halothane were sacrificed immediately after being removed from the chamber, which was 3 minutes after the onset of induction of anesthesia. Every anesthetized animal had loss of

the righting reflex and loss of withdrawal of the hind foot in response to pin prick.

The tissue level of pentobarbital in the optic nerves was determined by injecting pentobarbital 2-^{14}C (New England Nuclear sp. act. 3.23 mCi/mM) iv into two other mice (sacrificed by decapitation 2 minutes after injection) and measuring the radioactivity in the combined optic nerves with liquid scintillation counting techniques.

Micrographs having the total magnification of 54,875× were analyzed by both authors. Each author independently examined half the micrographs in each experimental group to reduce experimental bias and serve as checks for similarity in structure identification. For analysis, 90 myelinated axons cut transversely in each group were picked on the basis of absence of any obvious fixation artifact and clarity of plasma membrane. All microtubules in each axon which were clearly recognizable were counted. Microfilaments in the same axons were also counted, but were harder to identify with absolute certainty on cross-section at that magnification. The long and short diameters of each axon as measured from the internal surface of the plasma membrane were determined to calculate the cross-sectional area, as well as to give a comparison of ellipticity between experimental groups. Planimetry of 35 consecutive axons was used to compare direct measurements of axonal cross-sectional area and calculation of that area from measured diameters. The results were expressed as microtubule density, which was calculated by dividing the number of microtubules in an axon by its cross-sectional area.

Results

No difference between microtubule populations in the mice deeply anesthetized with pentobarbital or halothane and the controls was observed. Our control mice showed a typical pattern of microtubule density in relation to axon size. The distributions of sizes of axons, analyzed between groups, were identical.

Microtubule densities were compared according to axonal cross-sectional areas (fig. 1). Control densities ranged from 176.2 ± 28.9 microtubules/μm^2 ($\pm$SE) for axons of 0.06–0.10-μm^2 cross-sectional area to 30.1 ± 19.5 microtubules/μm^2 for axons of 1.00–2.50-μm^2 cross-sectional area. The values for pentobarbital-treated mice ranged from 154.9 ± 23.6 microtubules/μm^2 for 0.06–0.10-μm^2 axons ($P > 0.8$ compared with control) to 37.7 ± 9.2 microtubules/μm^2 for 1.00–2.50-μm^2 axons; for halothane-treated mice the microtubule density of 0.06–0.10-μm^2 axons was 177.2 ± 12.6 and that of 1.00–2.50-μm^2 axons, 32.0 ± 6.1. All P values, except as noted above, were >0.9 for experimental groups compared with control.

Microtubule/microfilament ratios (fig. 2) showed the greatest variation, probably owing to the difficulty of identifying filaments in cross-sections at that magnification. Microtubule/microfilament ratios were slightly higher for axons with the smallest cross-sectional diameters than for larger axons. In the axonal range 0.41–0.60 μm^2, the ratio of microtubules to microfilaments was significantly lower for halothane than for either the control condition or pentobarbital, but this may have resulted from the smaller sample of axons of this size.

The total axonal areas analyzed were 35.8 μm^2 for the control group, 29.8 μm^2 for the halothane-treated group, and 32.1 μm^2 for the pentobarbital-treated group, although the total area of micrograph examined was approximately 225 μm^2 for each group. Axonal areas measured with planimetry were comparable to calculated areas (correlation coefficient +0.927). The ellipticities of the axons in cross-section were comparable in all groups as measured by the ratios of short diameter to long diameter (0.66 ± 0.10 for the control group, 0.64 ± 0.09 for the pentobarbital-treated group, and 0.72 ± 0.12 for the halotrane-treated group; mean ratio of short/long diameter, $P > 0.9$ compared with control).

The level of pentobarbital in the optic nerves was 29.6 μg/g tissue wet weight, while brain levels amounted to 39 μg/g wet weight. A brain level of 29.6 μg/g is capable of producing narcosis in mice.[4]

Discussion

We were unable to demonstrate any significant differences between microtubule densities in axons of any size in optic nerves of mice deeply anesthetized with pentobarbital or

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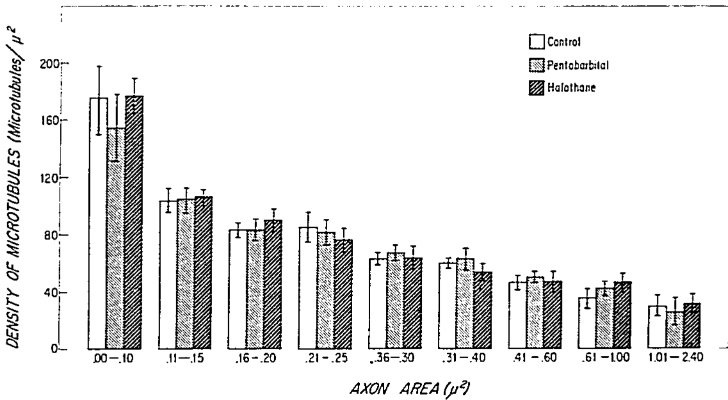

FIG. 1. Densities of microtubules in relation to axon sizes of optic nerves from adult mice given pentobarbital (40 mg/kg iv), halothane (1.5 per cent concentration by volume of inhaled vapor), and no anesthetic. No statistical difference between any of the groups was seen. Values are means ±SE.

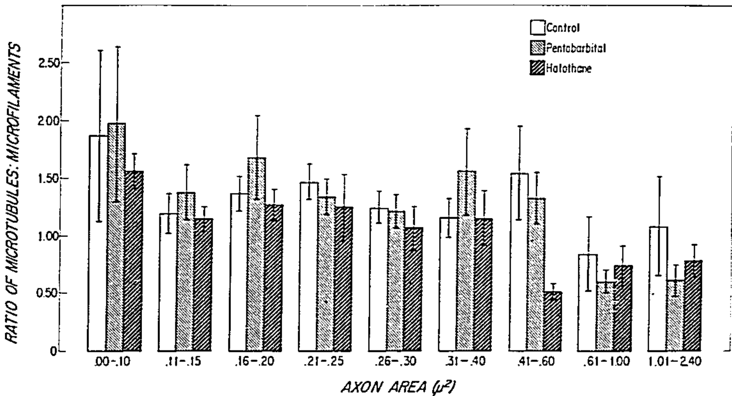

FIG. 2. This histogram shows microtubule-to-microfilament ratios in relation to mouse optic-nerve axonal cross-sectional areas for mice given pentobarbital (40 mg/kg iv), halothane (1.5 per cent), and no anesthetic. The ratio for halothane in the axon size range 0.41–0.60 μm^2 was significantly lower ($P < 0.01$) than control, but this may have been owing to the small sample of axons in this size range. Considerable variation is apparent in these ratios, in part because of difficulty in identifying microfilaments on cross-section with certainty.

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halothane and those of unanesthetized mice. This observation appears to contradict the Allison-Nunn hypothesis that the mechanism of anesthesia is the reversible depolymerization of microtubules. Thus, our data demonstrate that general anesthetics can produce narcosis without disappearance of neuronal microtubules.

The exact function of neuronal microtubules is not yet understood, and there is much speculation upon their function. That agents such as the vinca alkaloids and colchicine[5] can cause peripheral neuropathies as well as loss of neuronal microtubules has led to the idea that neuronal microtubules may be necessary for proper nerve function. Also, many agents capable of reversibly depolymerizing microtubules in certain systems are able to produce general anesthesia.[6-8] In this light, and based upon other circumstantial evidence,[9,10] Allison and Nunn proposed their hypothesis. To support this hypothesis, Allison *et al.*[2] demonstrated the reversible disappearance of microtubules in *Actinosphaerium* axopods with many common anesthetics at clinical concentrations. Recently their hypothesis was challenged by Fink and Kennedy,[11-13] who failed to find inhibition of rapid axonal transport in rabbit optic nerve (a process thought possibly to be related to neuronal microtubule function) upon exposure to halothane. The observation that high pressure (known to depolymerize microtubules in certain systems[6]) can reverse narcosis in newts,[14] instead of potentiating it as predicted by the Allison-Nunn hypothesis, also lends evidence against it. Our experiment using mouse optic-nerve microtubule densities in relation to axon size has directly tested the effects of pentobarbital and halothane on a specific microtubule system in a mammalian CNS under clinical conditions. We have produced in our mice a level of anesthesia that was clearly "surgical," and the amount of pentobarbital actually present in the optic nerve in itself was sufficient for narcosis.[4] We expect that this was true for halothane as well.[15]

Although the duration of exposure to anesthetics for our mice was relatively short, microtubule disappearance was seen in *Actinosphaerium* within the first 10 minutes.[2] This disappearance was quite rapid, such that two minutes after exposure to 2 per cent halothane, the mean axopodal length had decreased nearly 30 per cent. If the induction of anesthesia in mice were due to microtubule depolymerization, we would have expected to see some microtubule disappearance at the time of sacrifice. It is possible that disappearance of microtubules may be a long-term effect of anesthesia, either on the cell or directly on microtubules, but the lack of effect of long exposure to halothane on rabbit optic nerves argues against this. This is further supported by the observation of Halevy *et al.*[16] that after two hours of exposure to hexobarbital (240–360 mg/kg total dose), examination of cortex and other brain regions with transmission electron microscopy showed no change caused by the anesthetic. They stated that "the structure, number and organization of microtubules in the neuronal processes and perikaryon cytoplasm were unchanged compared to normals. . . ."

Although there are no quantitative data on mammalian optic-nerve microtubule densities, our control values were nearly identical to values obtained for mature chick optic nerve.[17] Furthermore, the distribution of microtubule density in relation to axon size is the same as that measured in mature chick optic nerves and described in rat optic nerves.[18]

It is possible that specific areas in the CNS contain microtubules which are of different protein composition, some of which are labile and sensitive to anesthetics and essential for maintenance of the awake state. This seems unlikely, since thus far only one microtubule protein with two distinct dimers has been isolated from mature mouse brain.[19] There is no evidence that microtubules in optic nerve respond to anesthetics in the same manner as microtubules in other locations of the CNS. However, the authors feel that the chemical and morphologic uniformity of microtubule protein in mouse brain, as well as the fact that the optic nerves are direct extensions of the CNS, located within the cranium and subject to blood flow similar to that in other parts of the CNS, argues against the possibility that microtubules in other areas of the brain and optic-nerve microtubules may respond differently.

To explain why our results contradict those of Allison *et al.*, we speculate that the recently demonstrated heterogeneity of micro-

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tubule subunit protein among different species[20,21] may be highly important, since it is known that slight differences in amino acid composition can alter the interaction of anesthetics with proteins.[22] Another interesting possibility relates to the apparent sensitivity to anesthetics of more ephemeral and mobile systems containing microtubules (flagella, cilia, mitotic spindles, and axopods).[23]

The results of this investigation demonstrate that microtubules in mouse optic nerve do not depolymerize during pentobarbital or halothane anesthesia, making the mechanism proposed by Allison and Nunn an unlikely explanation of the molecular basis of anesthesia. Our work lends further support to the concept of functionally different microtubules in different species, in spite of apparent morphologic and chemical similarities.

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