THE EFFECTS OF HALOTHANE ON CULTURED MOUSE NEUROBLASTOMA CELLS

I. Inhibition of Morphological Differentiation

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ABSTRACT

Mouse neuroblastoma cells (clone NB2a) were cultured in the presence of 0.3–2.1% halothane in the gas phase for up to 72 h. Halothane inhibited neurite extension dose dependently and virtually abolished microspike formation even at the lowest concentration tested. These effects were completely reversible. Electron microscopy demonstrated that microfilaments measuring 40–80 Å in diameter are the only fibrous organelles visible within microspikes. When the cells were exposed to halothane, no microfilamentous complexes could be identified in any cells and the subcortical regions of neurites often appeared devoid of individual microfilaments. Microtubules were still present in neurites after exposure to halothane concentrations at which microfilaments disappeared. However, at concentrations above 1.0%, microtubules gradually appeared to decrease in number. Short-term experiments showed that existing neurites and microspikes rapidly retracted when suddenly exposed to culture medium equilibrated with 1.0% halothane and quickly reformed when the halothane was removed. The inhibition of neuroblastoma cell differentiation by halothane appears to be mediated by disruption of 40–80 Å diameter microfilaments.

Halothane, a commonly used volatile anesthetic, has been shown to disrupt reversibly the mitotic apparatus (24), to cause retraction of protozoan axonemes (1), and to inhibit cell motility (24, 34). Enlarged microtubular structures measuring about 420 Å in diameter have been observed in protozoan axonemes (1) and isolated crayfish axons (13) after halothane treatment. These "macrotubular" structures are thought to represent neopolymerized forms of microtubular protein since they are altered by vinblastine and are colchicine labile (13). Collectively, these observations suggest that halothane can reversibly interfere with fundamental mechanisms responsible for intracellular motility and cellular translocation and can interact with at least some species of microtubule protein (tubulin) to produce alterations of microtubular structure. However, from a morphological standpoint, halothane-induced alterations in peripheral nerve preparations appear highly variable. For example, in isolated rabbit vagus nerves, halothane caused an increase in the number of typical microtubules (12); whereas in isolated crayfish axons, it resulted in a change from typical to macrotubular morphology (13). However, Fink and Kennedy (10) have reported that clinical concentrations of halothane have little effect on the rate of fast axonal transport or on microtubule structure or number in rabbit vagus nerves.

In an attempt to reconcile these observations

and to define fundamental structural and functional changes in neurons after anesthetic treatment, we have grown mouse neuroblastoma cells in the presence of halothane and have observed subsequent effects on cell differentiation, cell division, and macromolecular synthesis. Although these cells resemble mature sympathetic neurons (2, 20, 25), they are neoplastic and should not be considered as exact structural equivalents of normal nerve cells. This report presents evidence in support of the notion that the reversible inhibition of neurite and microspike formation by halothane is mediated by disruption of 40–80 Å diameter microfilaments.

MATERIALS AND METHODS

General

Stock and experimental cultures of mouse neuroblastoma cells (clone NB2a) were maintained at 37°C in Dulbecco's modified Eagle's medium containing 10% newborn calf serum (gamma globulin-free; Grand Island Biological Co., Grand Island, N. Y.). The cells were routinely grown in 60- or 100-mm plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Halothane (1,1,1-trifluoro, 2-bromo, 2-chloroethane) was a gift from Ayerst Laboratories (New York).

Two identical incubators were used in these experiments. One incubator was perfused with 5% CO_2 in air and provided the control atmosphere. The other incubator was perfused with halothane vaporized into a low flow of 95% air-5% CO_2 passing through a Copper Kettle vaporizer (Foregger Co., Inc., Smithtown, N. Y.) and subsequently into the incubator through copper tubing. By adjusting the air-CO_2 flow through the Copper Kettle vaporizer, concentrations of halothane in the incubator could be regulated and maintained over the range of concentrations tested (0.3–2.1% in the gas phase). In addition, halothane concentrations in the incubator were verified periodically by gas chromatography. Oxygen levels in both incubators were monitored routinely with a Beckman oxygen analyzer (Beckman Instruments, Inc., Fullerton, Calif.), and the concentration of CO_2 in the gas phase was determined by gas chromatography. In order to determine that halothane equilibrated with the culture medium, the halothane concentration in the liquid phase was also measured by gas chromatography. The medium was found to equilibrate with halothane within 2 h after placing the culture dishes in the halothane incubator.

Experimental

Replicate series of neuroblastoma cultures were established and allowed to attach to the growth surface for 16–20 h before being placed in the control or halothane-perfused incubators. Plates were inoculated with a number of cells such that the cultures were still in the log phase of growth after 72 h; the inoculum was usually 5×10^4 cells/60-mm culture dish containing 7 ml of medium. To obtain quantitative data on the proportion of cells with neurites, random fields of cultures were photographed after 24, 48, and 72 h in the control or halothane-perfused incubator. The number of cells possessing neurites (defined here as cells having one or more extensions with lengths at least equal to the diameter of the cell body) was determined and compared with the total number of cells in each field. This procedure was repeated for each concentration of halothane tested. Parallel control and halothane-treated cultures were examined by Nomarski optics and photographed using H and W Control film (H and W Company, St. Johnsbury, Vt.). Other control and halothane-exposed cultures were fixed for electron microscopy in 3% glutaraldehyde-0.05 M cacodylate buffer (pH 7.35), postfixed in 1.0% osmium tetroxide-cacodylate buffer, and embedded *in situ* according to the method of Brinkley et al. (5). Sections were cut on a Sorvall MT-2 ultramicrotome, stained with ethanolic uranyl acetate and lead citrate, and observed in an Hitachi 11-F electron microscope.

RESULTS

The mouse neuroblastoma cells in culture quickly attached to the growth surface and rapidly extended lengthy neurites and collateral microspikes which formed complex arborizational patterns

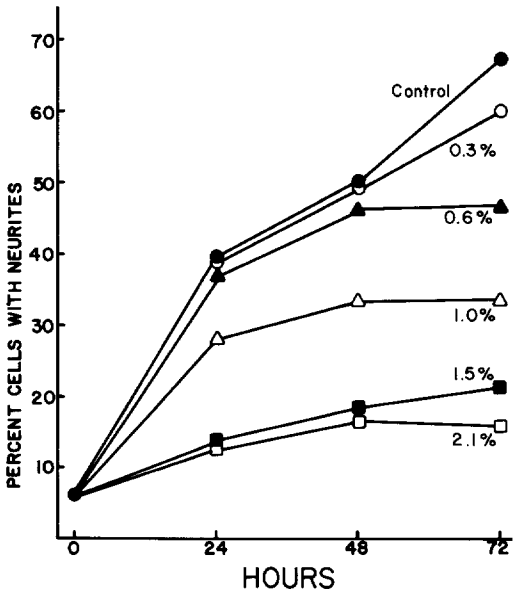
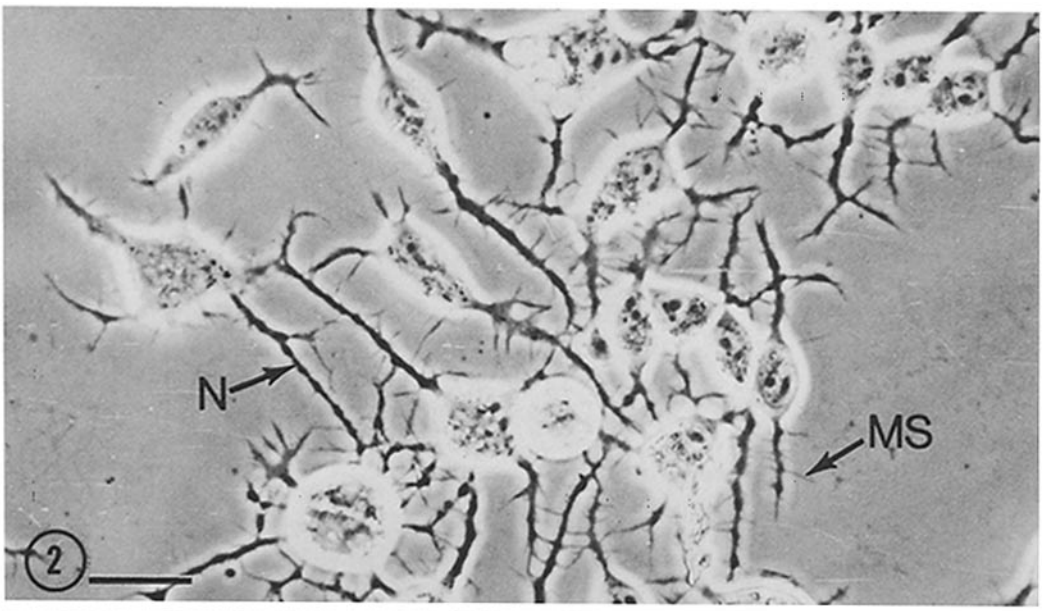
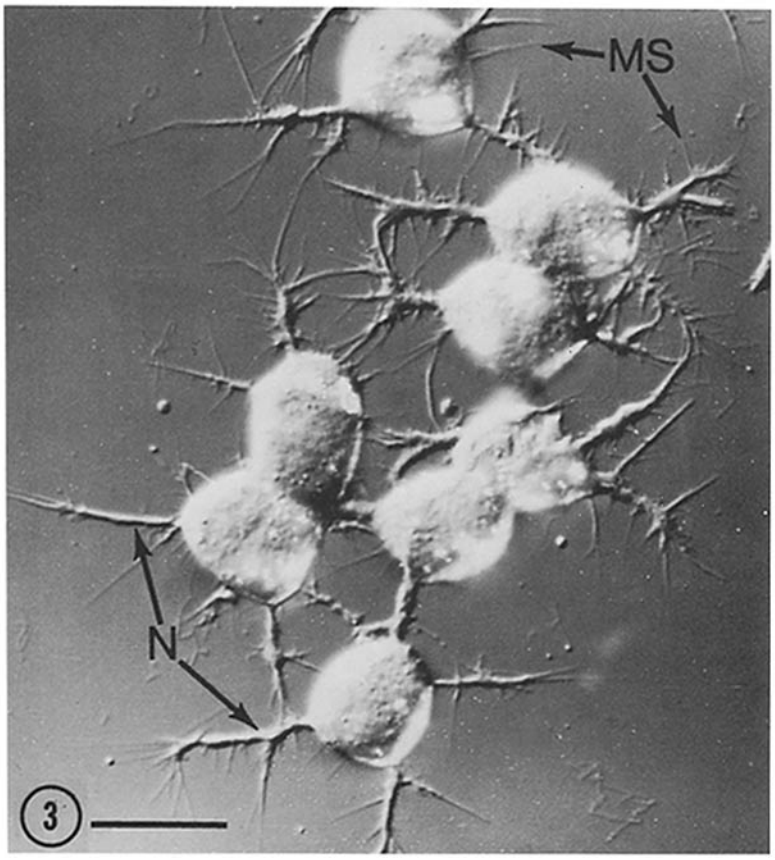


FIGURE 1 Effects of various halothane concentrations on the proportion of neuroblastoma cells (clone NB2a) possessing neurites. Each point is an average of three separate experiments and was determined from observations on at least 2,000 cells.





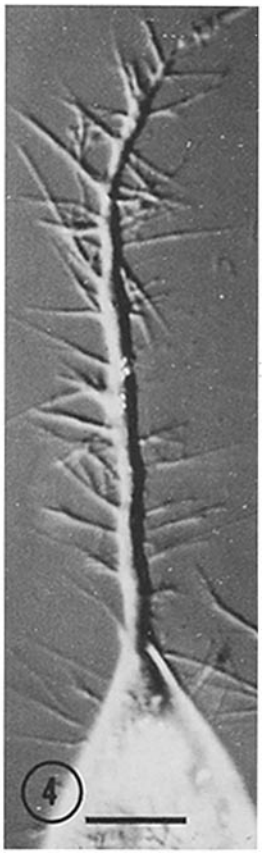
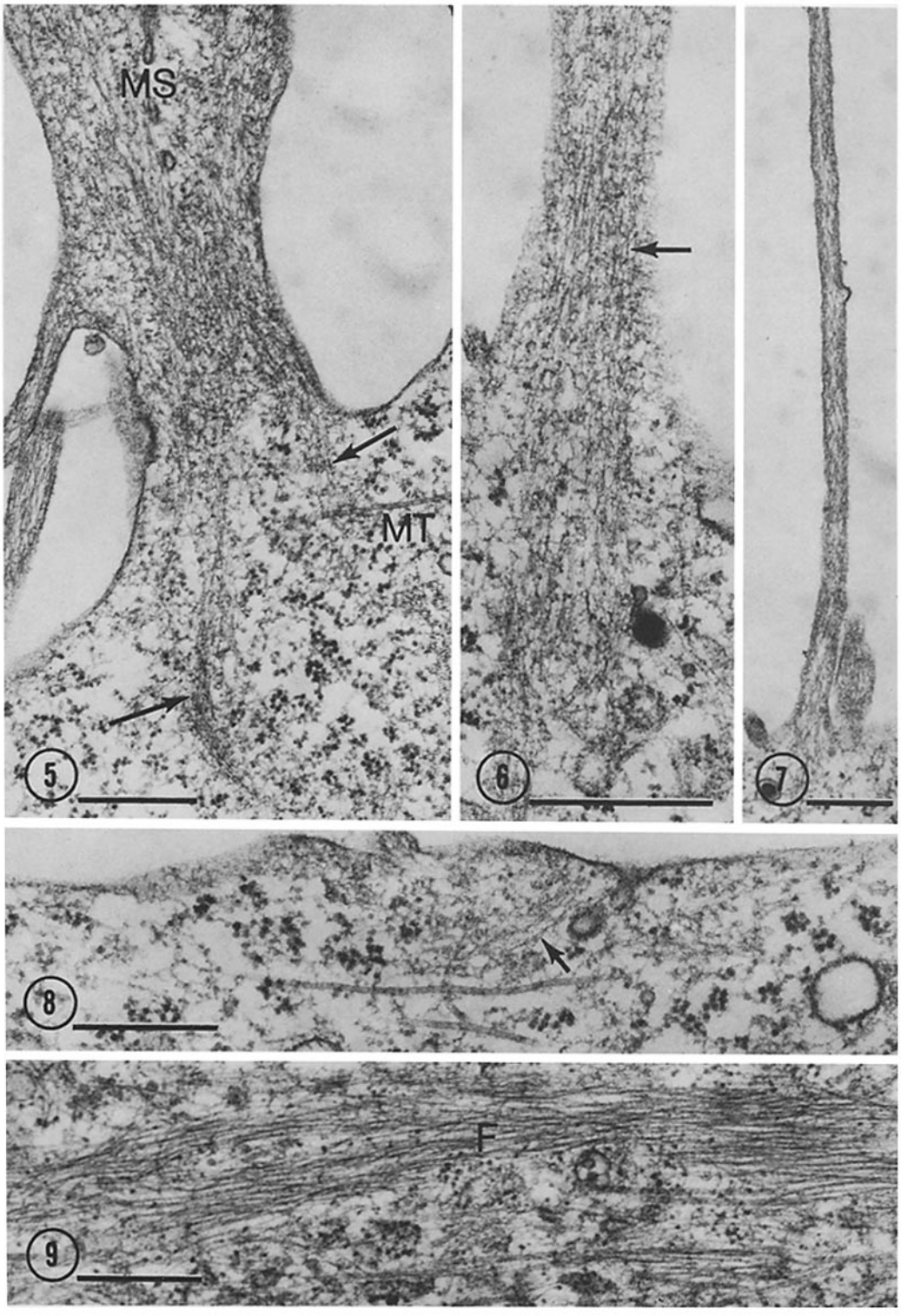


FIGURE 2 Conventional phase micrograph of a 72-h control culture. Note the neurites (*N*) extending from the cells and the smaller microspikes (*MS*) extending obliquely from the neurites and forming complex arborizational patterns on the growth surface between neighboring cells. Bar, 20 μm. × 670.

FIGURE 3 Nomarski micrograph of a 48-h control culture. The delicate appearance and relative abundance of microspikes (*MS*) are shown to greater advantage in these preparations. Neurites (*N*). Bar, 20 μm. × 900.

FIGURE 4 Nomarski micrograph of a single neurite extending from a 48-h control cell. Microspikes measuring up to about 20 μm in length radiate from the neurite and cell body. Bar, 10 μm. × 1,360.


MS
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between neighboring cells (Figs. 2–4). Neurites commonly contained typical 250-Å diameter microtubules of indeterminant length, an axial bundle of 100-Å filaments (neurofilaments), and numerous microfilaments measuring 40–80 Å in diameter and located primarily in the subcortical regions of neurites (Figs. 8, 9). Microfilaments were the only fibrous elements visible in microspikes and were oriented, for the most part, parallel to the major axis of the microspike (Figs. 5–7). In several sections, microfilamentous complexes found within microspikes extended into the neurites and terminated in close proximity to microtubule profiles that were oriented parallel to the axis of the neurite (Fig. 5).

Halothane inhibited neurite extension in a dose-dependent fashion over the entire range of concentrations tested (Fig. 1). At low concentrations (0.3–0.6% halothane in the gas phase), reductions in the number of neurites were most evident after 48–72 h of incubation. At halothane concentrations of 1.0% and higher, reductions in neurite formation were evident within 24 h.

Halothane severely inhibited microspike formation (Figs. 10–12). Even low concentrations of halothane, which had minimal effects on neurite formation (0.3–0.6%), virtually abolished microspike formation. Cells that retained lengthy neurites after halothane treatment were devoid of microspikes and, instead, possessed only short rudimentary processes or surface irregularities (Fig. 12).

To test the reversibility of these effects on neurite extension and microspike formation by halothane, cultures were grown in the presence of 1.5–2.1% halothane for 24–48 h to inhibit neurite and microspike formation. These cultures were then placed in the control incubator after exchanging the halothane-equilibrated medium in the culture dishes for control medium. Within 12–24 h, cells treated in this manner were indistinguishable from control cells and possessed typical neurites and microspikes. The effects of halothane on existing neurites and microspikes were determined by growing cultures in the control atmosphere for 24–48 h to allow neurites and microspikes to form. The medium in each culture was then replaced with medium pre-equilibrated with 1.0% halothane. Both neurites and microspikes began retracting within 10–15 min after the exchange of medium. When the halothane-equilibrated medium was replaced with control medium, neurites and microspikes gradually reappeared and after 12–24 h these cultures resembled control cultures.

Halothane-treated cells were examined for any fine structural changes that might be responsible for neurite and microspike sensitivity to halothane. Few microfilaments could be identified in the subcortical regions of neurites or cell bodies after exposure even to low concentrations of halothane. Furthermore, no microfilamentous complexes associated with membranes or microtubules were encountered in any section of halothane-treated cells (Fig. 13). Typical microtubular profiles were still present in neurites remaining after halothane treatment but their numbers appeared to decrease in cells incubated in the presence of halothane concentrations above 1.0–1.5%. The axial bundle of 100-Å filaments often appeared tangled and disorganized (Fig. 14). Except for these changes in the fibrous organelles and slight swelling of mitochondria, few other consistent changes in cell fine structure were observed. However, at higher concentrations of halothane (1.5–2.1%), surviving neurites contained increased numbers of membranous organelles including lipid inclusions, bizarrely shaped mitochondria, and dilated vacuoles of endoplasmic reticulum (Fig. 13).

FIGURES 5–7 Electron micrographs of three longitudinally sectioned microspikes (*MS*) extending from cells grown in control atmospheres for 72 h. Microfilaments measuring 40–80 Å in diameter (arrows) are the only fibrous organelles present in microspikes. In several cases, as in Fig. 5, bundles of microfilaments extended into the neurites and sometimes ended in close association with neurite microtubules (*MT*). Bars, 0.5 μm. Fig. 5, × 36,100; Fig. 6, × 56,500; Fig. 7, × 25,750.

FIGURE 8 Longitudinal section through the subcortical region of a 72-h control neurite. Subcortical microfilaments are visible (arrow) as are microtubular profiles. Bar, 0.5 μm. × 44,000.

FIGURE 9 An axial bundle of 100-Å filaments (*F*) was often seen in the core of neurites along with microtubules. Bar, 0.5 μm. × 38,000.

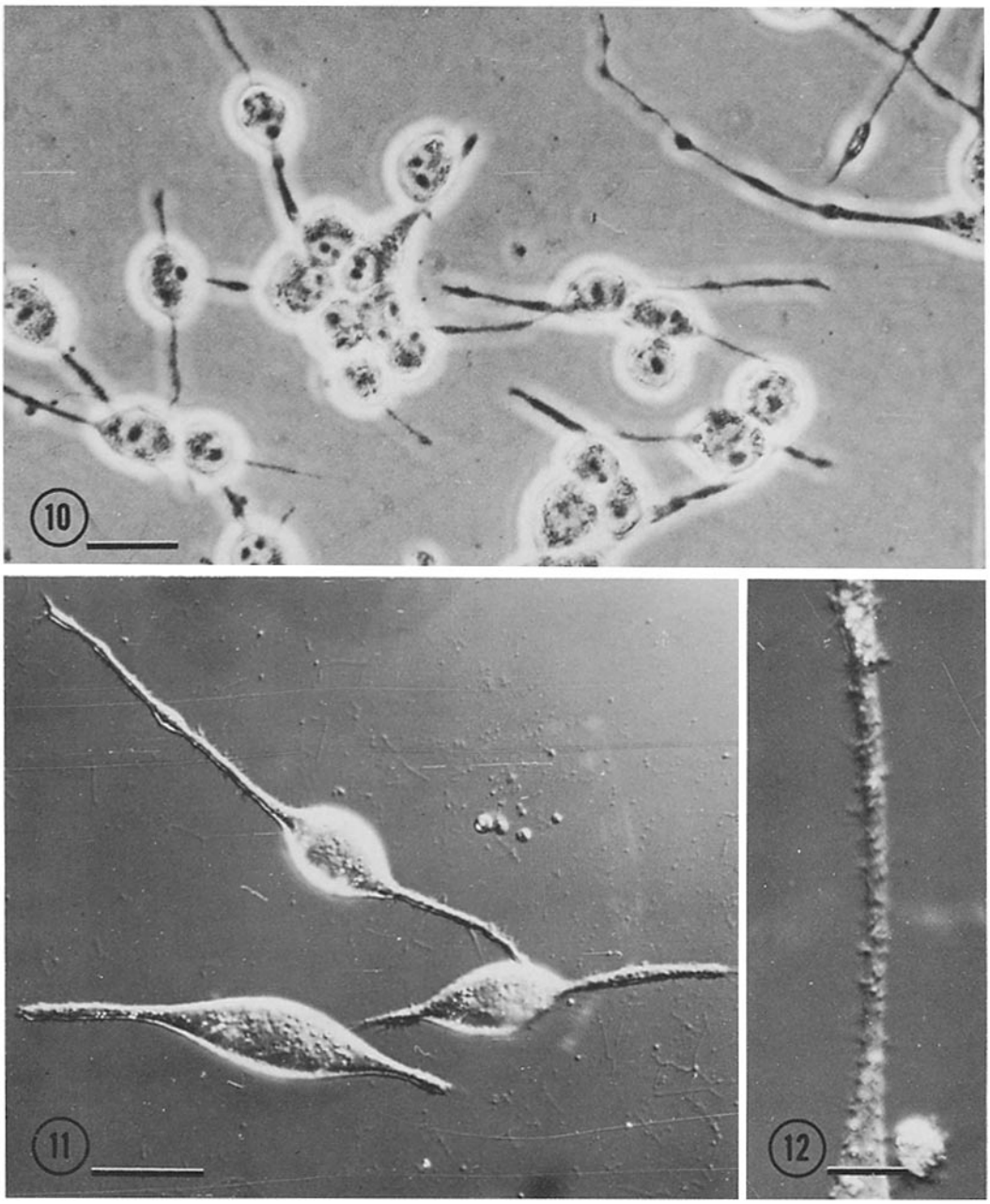

FIGURE 10 Phase micrograph of a neuroblastoma culture exposed to 1.0% halothane for 72 h. This field was selected for the presence of neurites to show the absence of microspikes. Compare with Fig. 2. Bar, 20 μm. × 650.

FIGURE 11 Nomarski micrograph of cells grown in the presence of 0.75% halothane for 72 h. This field was also selected for the presence of neurites to illustrate the virtual absence of microspikes. Compare with Fig. 3. Bar, 20 μm. × 780.

FIGURE 12 High magnification Nomarski micrograph of a neurite exposed to 0.75% halothane for 72 h. Compare with Fig. 4. Lengthy microspikes were absent but surface irregularities were often seen. Bar, 10 μm. × 1,120.

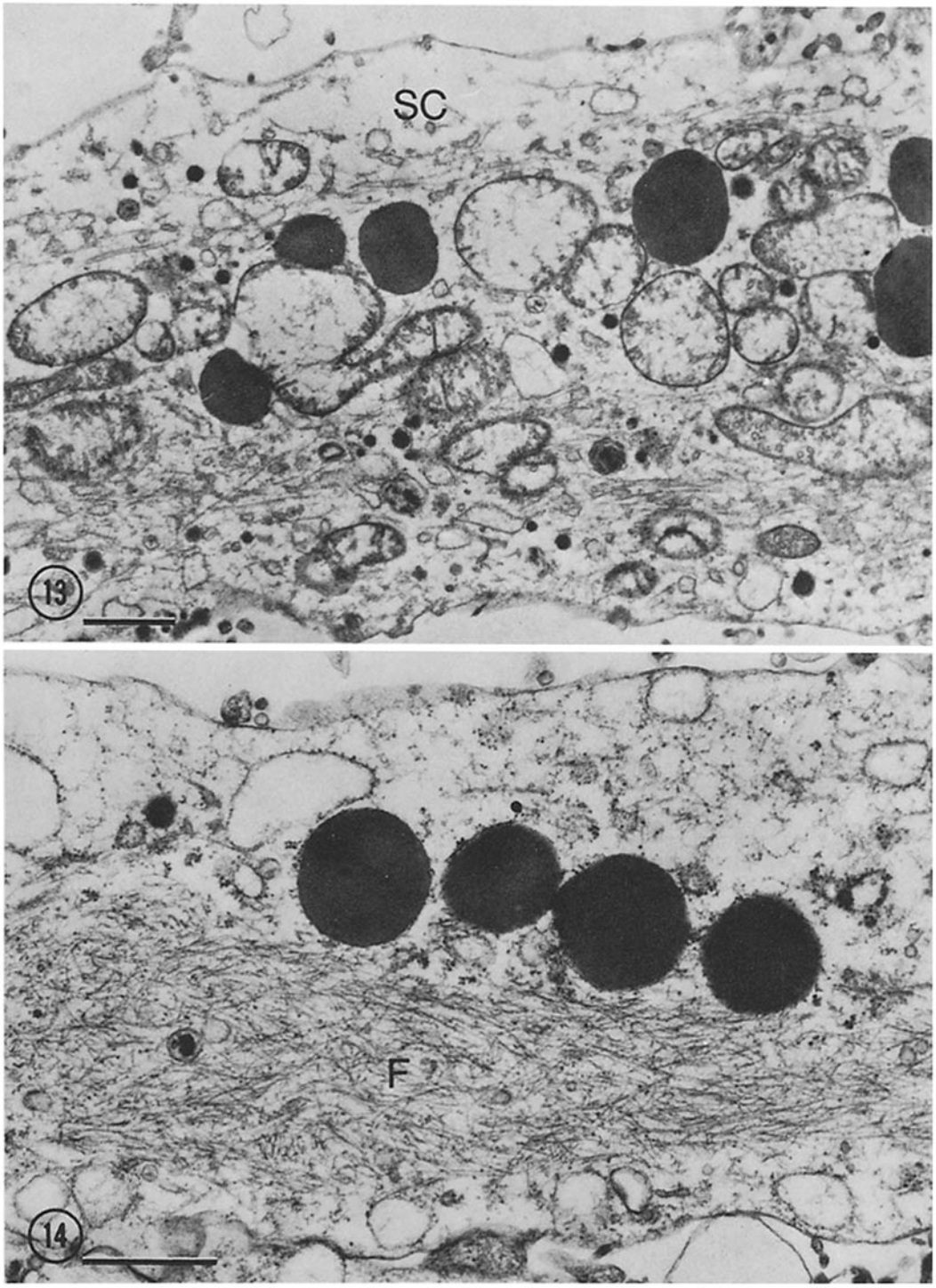

FIGURE 13 Longitudinal section through a neurite exposed to 1.5% halothane for 48 h. The subcortical regions (*SC*) were often clear and few microfilaments could be identified in any preparations. Numerous mitochondria, dense-core granules, and lipid inclusions were frequently observed throughout the entire length of such neurites. Bar, 1.0 μm. × 13,500.

FIGURE 14 Section through a neurite exposed to 1.0% halothane for 48 h showing the tangled appearance of the axial filaments (*F*). No microfilamentous complexes were encountered in any of these sections but typical microtubular profiles were still evident. Bar, 1.0 μm. × 20,100.

DISCUSSION

The observations that halothane reversibly inhibits neurite extension and microspike formation are generally consistent with observations demonstrating halothane-induced retraction of *Actinosphærium* axonemes (1) and cessation of *Dictyostelium* mobility and pseudopod formation (34). These results suggest that halothane transiently interferes with mechanisms or structures which are essential for the maintenance of cell shape and intimately associated with intracellular movement. Considerable evidence has accumulated implicating microtubules and particularly microfilaments in these cellular activities.

Roisen and Rebhun (28) and Daniels (8) have shown that microtubular assembly is intimately associated with elongation of nerve cell processes, and Prasad (26), using cytochalasin B and vinblastine, concluded that microtubules and microfilaments are equally important for neurite extension. More recently, Roisen and Murphy (27), using combinations of cytochalasin B and Colcemid, have shown that both microtubules and microfilaments are required for the maintenance of neurites extending from explanted chick dorsal root ganglia. Thus, assembly of microtubules and microfilaments appears to be necessary for neurite extension, and intact microtubules and microfilaments are required for maintenance of neurite structure. Even though intact microtubules are present in neurites surviving exposure to low concentrations of halothane, microfilamentous complexes are absent and only a few individual microfilaments can be identified in the subcortical regions of neurites. Accordingly, in view of these results and of the apparent interdependency of microtubules and microfilaments in extension and maintenance of neurite structure, it may be possible that the failure of neurites to form in the presence of halothane may be due to an uncoupling of the microtubule-microfilament "complex" by disruption of the microfilamentous component. The retraction of preformed neurites upon exposure to halothane may also reflect a disruption of microfilament structure, especially since neurite maintenance is thought to depend, in part, on an intact microfilament system. However, it is also possible that halothane simultaneously inhibits normal microtubular assembly and, at sufficient concentrations, can lead to microtubular disassembly. This possibility must be considered in view of experiments demonstrating microtubular depolymerization in protozoan axonemes (1), crayfish axons (13), and spindles of fertilized *Echinus* eggs (23) exposed to halothane. However, the concentrations of halothane that have been shown to cause microtubular depolymerization generally exceed the concentrations reported here (as low as 0.3%) to disrupt microfilament structure. Therefore, at least in neuroblastoma cells, microfilaments and perhaps their ability to organize into functional complexes are more sensitive to halothane than are microtubules. This suggestion is strengthened by the observations that microspike formation was virtually prevented at halothane concentrations having little immediate effect on neurite extension and that neurites surviving exposure to low halothane concentrations possessed structurally intact microtubules, but few microfilaments.

Microfilaments measuring 40–80Å in diameter have been reported in a variety of cell types and have been localized within actively motile areas of cells including cleavage furrows (29), undulating membranes (31), and elongating nerve growth cones and microspikes (35, 36). In these structures, microfilaments form complex three-dimensional networks, sometimes in close association with nearby microtubules. Microfilaments are thought to have an actin-like composition since they can be decorated with heavy meromyosin (HMM) (15) and can be made to undergo reversible actin-like F (filament)-G (monomeric) transformations when isolated (37). Due to their location in actively motile structures and their actin-like nature, microfilaments are thought to have a contractile function and may be responsible, at least in part, for subcellular movements and cellular translocations (4, 19, 31). The presence and distribution of HMM-binding microfilaments in cultured mouse neuroblastoma cells has been reported by Burton and Kirkland (6) and Chang and Goldman (7), and has been confirmed by us in recent experiments describing the presence of HMM-binding microfilaments in the mitotic apparatus of neuroblastoma cells (14). Microfilaments in the subcortical regions of neuroblastoma cells and neurites, as well as those visible in microspikes, bind HMM.

If these microfilaments, which are the only structural organelles visible in microspikes, have in fact an actin-like character and can undergo reversible F-to-G structural transformations, one possible explanation of our results is that halothane may in some way facilitate an F (filament)-to-G (monomeric) conversion of microfilament

structure. A conversion to the monomeric form would account for the relative paucity of intact microfilaments after halothane treatment. The reverse (G to F) transformation would explain the sudden resumption of microspike formation and neurite elongation concomitant with microfilament reappearance, after the removal of halothane from the growth medium. Our preliminary observations, showing that the amount of identifiable HMM-binding material in halothane-treated cells is greatly reduced in comparison with that visible in cells grown in control atmospheres, provide additional evidence for a halothane-induced F-to-G disassembly of microfilament structure.

The action of halothane resembles that of cytochalasin B in that both agents inhibit cell motility and phenotypic differentiation of cells in culture. For example, cytochalasin B inhibits cell motility (11, 31, 32), and halothane has been shown to reversibly inhibit the motility of protozoans (22) and lymphocytes (24). Both halothane and cytochalasin B have identical inhibitory effects on *Dictyostelium* mobility and pseudopod formation (34). Moreover, cytochalasin B (27, 36), and now halothane, have been shown to inhibit neurite elongation and microspike formation in neurally derived cells. Both agents, although chemically very different, appear to have a common mode of action, at least so far, as they disrupt microfilament structure or alter the organizational complexity or functionality of microfilamentous systems. Spooner (30) has recently proposed that the primary site of action of cytochalasin B is at the cell surface, and microfilaments or microfilamentous complexes might be altered by modification of their insertion points into the plasma membrane. However, halothane inhibits karyokinesis, apparently by disrupting microfilaments which are located within the mitotic apparatus of dividing cells (14). Cytochalasin B, on the other hand, appears to have little effect on chromosome separation (16, 21). Since mitotic microfilaments which are disrupted by halothane are not attached to membranes, halothane may be capable of interacting directly with the protein subunits of the microfilaments within the cell to bring about microfilament disruption. Certainly, an alternative explanation is that halothane may disrupt microfilament structure directly by altering the integrity of the plasma membrane. However, there is good evidence that volatile anesthetics, including halothane, can induce reversible conformational changes in globular proteins, most likely by binding to the protein moieties through hydrophobic forces (3, 17, 18, 33, see reference 9 for a review). Binding of halothane to microtubular subunits (tubulin) has been invoked as a possible mechanism to explain the formation of "macrotubules" measuring 420 Å through an alteration of the bonding angle between tubulin subunits (13). Assuming that microfilaments are composed of actin-like monomers organized into filaments, we suggest that reversible conformational changes induced by halothane may transiently render the monomeric subunits incapable of maintaining or forming subunit-subunit interactions.

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