Anesthesiology
79:1338–1347, 1993

Effects of Inhalational Anesthetics on Biochemical Events in Growing Neuronal Tips

Shigeru Saito, M.D., Tatsushi Fujita, M.D.,† Michihiro Igarashi, M.D.‡*

Background: **The influence of general anesthetics on developing organs has been a source of concern for many years. The central nervous system, which is developing rapidly at the time of birth, is of special interest in this regard. In this study, the biochemical characteristics of developing neural tips (growth cones) were examined after exposure to anesthetics to elucidate the molecular mechanism by which long-lasting alterations in the nervous system, including neuroteratogenicity, as previously described, were evoked.**

Methods: **Neonatal rats were exposed to an atmosphere containing inhalational anesthetics (1% halothane or 75% nitrous oxide) or a control atmosphere (25% O_2 and 75% N_2) for 6 h at postnatal day 1. After this exposure, growth cone particles were isolated from the forebrain using a recently devised cell fractionation method at postnatal days 2, 3, 4, and 5. Protein composition, phosphoprotein patterns, and protein kinase C (PKC) activities of the isolated growth cones were compared between each group exposed to anesthetics and the control group. The dose–response relationship of the action of anesthetics on PKC activity was also examined (at 0.5 and 0.75% halothane and 25 and 50% N_2O).**

Results: **The increase in body weight and brain wet weight were not significantly affected by exposure to either anesthetic. No apparent influence on protein composition was observed by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). However, calcium-dependent protein phosphorylation of the 46 kDa protein and of the 80 kDa protein, which is reported to be mediated by PKC, were significantly reduced after exposure to the anesthetics. A direct assay of PKC activity in growth cone particles indicated that PKC activity in the growth cone was 70.6 ± 9.6% of the control value at 24 h after exposure to 1% halothane, and 63.2 ± 4.9% after exposure to 75% nitrous oxide. Exposure to 0.75% halothane or 50% nitrous oxide had a similar, but lesser, effect on this parameter. In contrast, exposure to 0.5% halothane or 25% nitrous oxide evoked no apparent effect. Thus, the PKC activity in growth cone particles, which is thought to play an important role in signal transduction in the developing brain, was shown to be affected by exposure to inhalational anesthetics over a range of concentrations.**

Conclusions: **Considering the crucial role of growth cones in the establishment of the neuronal network, the interruption of signal transduction in the growth cone at a time that is critical in directing the neurite extension may evoke a long-lasting alteration in the neural network. Therefore, the effect of inhalational anesthetics on the growth cone enzyme observed in this study may have a major role in the mechanism that induces morphologic or behavioral neuroabnormalities in later life. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: halothane. Brain: development; growth cone; synaptogenesis. Toxicity: anesthetics; teratogenicity.)**

This article is accompanied by a Highlight. Please see this issue of ANESTHESIOLOGY, page 28A.

* Instructor, Department of Anesthesiology and Reanimatology.

† Professor and Chairman, Department of Anesthesiology and Reanimatology.

‡ Instructor, Department of Molecular and Cellular Neurobiology.

Received from the Department of Anesthesiology and Reanimatology, Department of Molecular and Cellular Neurobiology, Gunma University School of Medicine, Gunma, Japan. Accepted for publication August 18, 1993.

Address reprint requests to Dr. Saito: Department of Anesthesiology, Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi-shi, Gunma, 371, Japan.

FOR many years, the effects of drugs on animal development have been a matter of great interest in medical science. In particular, the effect of drugs on the central nervous system, which is undergoing rapid development at the time of birth, is a topic of investigation by clinicians. In the field of anesthesiology, some information has been obtained, mainly from morphology or behavioral science, indicating that perinatal exposure to general anesthetics has some significant effects on synaptogenesis in the developing brain,[1] and, thereafter, on behavioral characteristics.[2–10]

In the developing brain, neurons have two kinds of elongating neurites, the axon and dendrite. The growth cones are located at the distal tips of both neurites, and they actively search for the precise pathway for future synaptogenesis in their target area. The unique biochemical characteristics of nerve growth cones are thought to be closely related to their role in pathway guidance of the developing neuron, and in the establishment of the neuronal network in the central nervous system.[11,12] Therefore, the investigation of molecular

events in the growth cones, in the presence or absence of drugs, is thought to be important in the elucidation of the biologic action of drugs on the developing nervous system. In 1983, Karl Pfenninger and Gordon-Weeks independently developed new biochemical techniques for the isolation of growth cones.[13,14] Using these isolation techniques, the molecular mechanism of neurite extension has been investigated biochemically, morphologically, and pharmacologically. With a slight modification of their techniques, we have also shown that growth cone particles or membranes, isolated biochemically, have unique characteristics in lipid composition[15,16] or in phosphoprotein composition.[17,18] Furthermore, we have reported that growth cones express muscarinic acetylcholine receptors before synaptogenesis.[19] Considering the crucial role of growth cones in the developing brain and their distinctive characteristics, it is plausible that exposure to anesthetics during the neonatal period, which has already been described as having a significant influence on synaptogenesis morphologically or behaviorally, also influences the biochemical characteristics of developing neural tips. In this study, to clarify the biochemical effects of anesthetics on growth cones, we examined the protein composition of isolated growth cones after exposure to anesthetics at postnatal day 1. A protein phosphorylation, which is reported to be essential in intracellular signal transduction,[20–22] was also examined in isolated growth cones.

Materials and Methods

Materials

The standard molecular mass markers and protein assay kits were obtained from Bio-Rad Laboratories (Richmond, VA). Silverstaining kits were purchased from Daiichi (Tokyo, Japan). [γ-^{32}P]-Labeled ATP was obtained from NEN (Boston, MA). Protein kinase C activity detection kits were obtained from Amersham (Amersham, Buckinghamshire, UK). Riaflor and protease inhibitors were purchased from Sigma (St. Louis, MO).

Subjects

All of the animal experiments were performed with the approval of the local ethical committee of Gunma University School of Medicine.

Wistar ST rats (SLC, Shizuoka, Japan) were exposed to anesthetics in a chamber (sized 35 × 35 × 25 cm) with an atmosphere of halothane (1% halothane in 75% N_2/25% O_2) or nitrous oxide (75% N_2O and 25% O_2) for 6 h at postnatal day 1. In a control group, rats were exposed to the same chamber with an atmosphere of 75% N_2 and 25% O_2. All other conditions were identical. To examine the dose–response relationship, other anesthetic concentrations (0.25% or 0.5% halothane in 75% N_2 and 25% O_2, 25% N_2O + 50% N_2 + 25% O_2 or 50% N_2O + 25% N_2 + 25% O_2) were used to obtain samples for the PKC assay. During exposure, the environment in the chamber was controlled using a heating mat and humidifier (floor temperature at 38° C, chamber temperature 26–30° C, humidity 50–60%), and the gas concentrations were calibrated intermittently by gas chromatography, and were maintained within ±5% of the intended value. Gas supply to the chamber was 3 l/min, and the CO_2 partial pressure, which was measured by a gas analyzer (ABL3; Radiometer, Copenhagen, Denmark) every 30 min, was kept below 0.2% throughout the experiments. As more than ten neonatal brains are necessary to obtain a sufficient amount of growth cone particle (GCP) protein for the following assays, all the pups from a litter (10–12 pups) were used for a single exposure experiment. The litter size ranged from 10 to 12, and there were no significant differences in litter size between groups. After exposure to the anesthetics or to the control atmosphere, pups were returned to their own dam and housed in a room with the dam until postnatal day 2–5. Diet and water were freely available. The time course of the experiment was followed using samples obtained from four time points after exposure; in addition, four independent experiments were performed to obtain single point of data for statistical analysis; therefore, 16 litters were independently exposed to each atmospheric condition. In preliminary experiments to assess the possibility of hypoxia or hypercarbia of rats during exposure to the anesthetics, the transcutaneous O_2 partial pressure ($tcPO_2$) and CO_2 partial pressure ($tcPCO_2$) were monitored (by MicroGas 7640; Kontron, Switzerland) every 30 min during a series of exposure experiments as described above (1% halothane, 75% N_2O, or control gas) using five P1 pups for each atmospheric condition.

Preparation of Growth Cone Particles

A litter of pups were decapitated at each previously scheduled date, *i.e.*, postnatal day 2 (P2), P3, P4, and P5, and were used in further preparations as follows.

The GCP fractions were isolated from neonatal rat forebrain, using the modified preparation methods previously described.[15] Briefly, neonatal rat forebrains were homogenized with eight volumes of 0.32 M sucrose containing 1 mM TES/NaOH (*p*H 7.3), 1 mM $MgCl_2$, and some protease inhibitors. After a low-speed centrifugation, the supernatant was loaded onto a 0.75 M/1.0 M sucrose gradient and centrifuged for 60 min at 141,000*g*. The interface between 0.32 M and 0.75 M sucrose (Pfenninger, s A-fraction) was collected, diluted with 0.32 M sucrose, and centrifuged for 90 min at 20,000*g*. The GCP fraction was obtained as a pellet. The pellet of GCP was homogenized and resuspended at 4° C with 6 mM Tris/HCl *p*H 8.1 for electrophoresis, or with 50 mM Tris/HCl *p*H 7.5 containing 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 10 mM benzamidine, and 50 μg/ml phenylmethylsulphonyl fluoride for PKC-activity assay.

Body weight and brain wet weight were measured before sample preparation. Protein concentrations were determined according to the method of Bradford,[23] using bovine plasma γ-globulin as a standard.

Electrophoresis and Phosphorylation Study

One-dimensional sodium dodecylsulfate-poly acrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli.[24] After electrophoresis, gels were stained with ammoniacal silver.

The protein phosphorylation study was performed as detailed previously,[17] using two types of reaction mixture. Reaction mixture A, for conditions in which tyrosine residues were preferentially phosphorylated, contained 20 mM HEPES (*p*H 7.5), 10 mM $MnCl_2$, 1 mM EGTA, 10 μM $ZnCl_2$, 30 μM $NaVO_3$, 0.1% Nonidet P-40, and 20–25 μg of GCP protein. Reaction mixture B, for Ca^{2+}-dependent phosphorylation, was composed of 20 mM HEPES (*p*H 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 20–25 μg of GCP proteins in a final volume of 50 μl. Under both conditions after a 1-min preincubation at 30° C, the phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP (2.22GBq/μmol) to 0.5 μM, followed by a 3-min incubation at 30° C. The reaction was terminated as described previously.[20] The phosphorylated proteins were electrophoresed and then autoradiographed. Film images were quantified with a scanning laser densitometer (LKB 2202).[16]

Protein Kinase C Activity Assay

Protein kinase C activities were examined following the PKC activity assay protocol designed by Amersham.[25] Briefly, 25 μl of component mixture, containing 3 mM calcium acetate, 6 μg/ml phorbol 12-myristate 13-acetate, 7.5 mM dithiothreitol, and 225 μM substrate peptide, was mixed with 25 μl of GCP sample solution containing 30–50 μg protein. The phosphorylation reaction was started by the addition of [γ-^{32}P]ATP to 50 μM (25 μl of magnesium [γ-^{32}P]ATP solution). After incubation at 25° C for 15 min, the reaction was terminated by the addition of 100 μl of stop reagent. An aliquot (125 μl in volume) was spotted onto a peptide binding paper and washed twice to remove radioactivity that was not incorporated into the peptide or endogenous protein. The spotted binding papers were placed in plastic minivials and dried, and 3 ml of the scintillation cocktail Reaflor was added. The radioactivity trapped on the filters was measured using a Aloca 650 liquid scintillation counter. Specific PKC activity in the growth cone fraction was defined as the difference between the activity in the presence and in the absence of GCP samples. To conserve the *in vivo* state of the kinase in this assay, the enzyme purification procedure was omitted. Therefore, the phosphorylation activity of the samples are expressed relative to the P2 control value. In the preliminary experiments, the linearity of a set amount of sample protein and the kinase activity value was confirmed using control GCP samples within the range used in this study. During all measurements, the assayist remained uninformed as to which group the assay sample was derived from, and, after the measurements, the data and the sample names were matched.

The data are expressed as mean ± SEM. The results of multiple groups were analyzed by one-way ANOVA, and comparisons between groups were assessed by Scheffe's test, using Statview II™ (Abacus Concepts, Berkeley, CA).

Results

During exposure, pups receiving 75% nitrous oxide or the control atmosphere were awake and active. The activity of those exposed to 1% halothane appeared to decrease, but they recovered when exposed to noise or shaking. When removed from the chamber and returned to their dam, the pups of all three groups began sucking vigorously. In pups exposed to the control gas, the transcutaneous O_2 partial pressure and the CO_2 partial pressure fluctuated between 89–128 mmHg (102.0 ± 16.0 mmHg, mean ± SD) and between 22–38 mmHg (31.8 ± 4.6 mmHg), respectively. In the

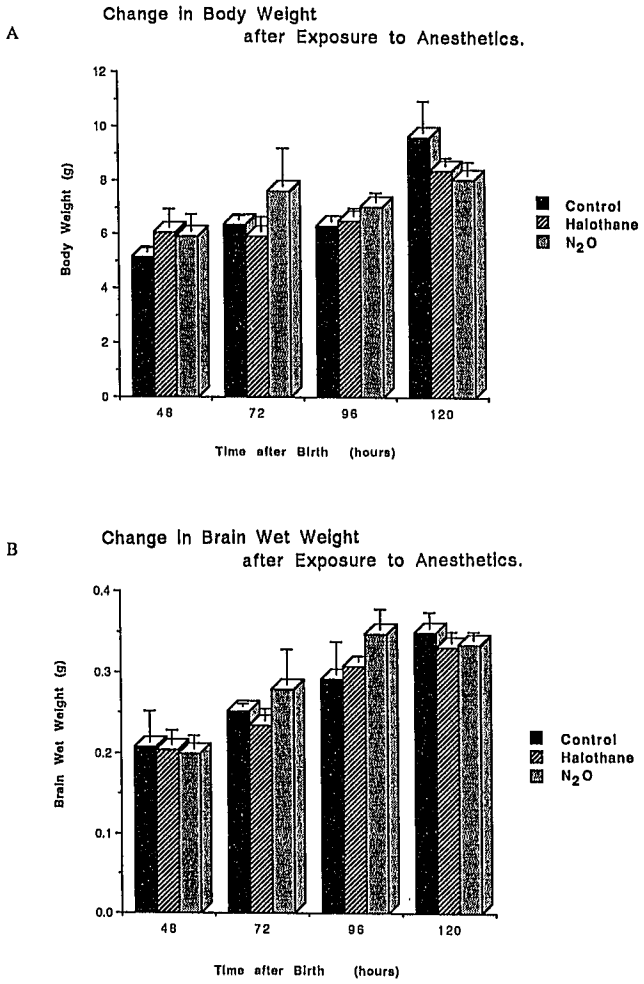

Fig. 1. Change in body weight (*A*) and brain wet weight (*B*) after exposure to 75% nitrous oxide or 1% halothane. Data are expressed as mean ± SEM. In both measurements, no significant differences were observed between the control group values and those of each group exposed to anesthetic at any date after birth.

group of pups exposed to anesthetic, the $tcPO_2$ fluctuated between 85–122 mmHg (101.1 ± 11.1 mmHg) in the 1% halothane group and between 87–114 mmHg (99.6 ± 8.5 mmHg) in the 75% nitrous oxide group, the $tcPCO_2$ were 25–41 mmHg (35.9 ± 4.2 mmHg) in the 1% halothane group and 24–43 mmHg (36.3 ± 4.9 mmHg) in the 75% nitrous oxide group. For $tcPO_2$ and $tcPCO_2$, no significant differences were observed between groups for each value ($P < 0.05$).

Among the three groups, no significant difference was observed in body weight and in brain wet weight (fig. 1). The protein composition and the tyrosine phosphorylation patterns of growth cone particle fractions were in agreement with our previous reports,[17,18,19] and no marked alterations in the gel band pattern were observed, even after exposure to the anesthetics (figs. 2 and 3, table 1). However, the Ca^{2+}-dependent phosphorylation pattern of growth cone particle proteins was shown to be affected by the exposure to the anesthetics (fig. 4), although the phosphorylation pattern of the control group was in agreement with our previous observations, in which animals were killed without deprivation of their dam, and without exposure to 75% N_2.[16,17,19] The most apparent alteration after exposure to anesthetics was a reduction of ^{32}P incorporation into the 46-kD and into the 80-kD proteins compared with the control group, especially at 24 h after exposure, namely at P2. These proteins are known to be specifically phosphorylated by protein kinase C.[26] Quantitative analysis by densitometry clearly demonstrated a reduction in ^{32}P incorporation into these two major phosphoproteins, as shown in table 2. Although several other phosphoprotein bands in the gel-autora-

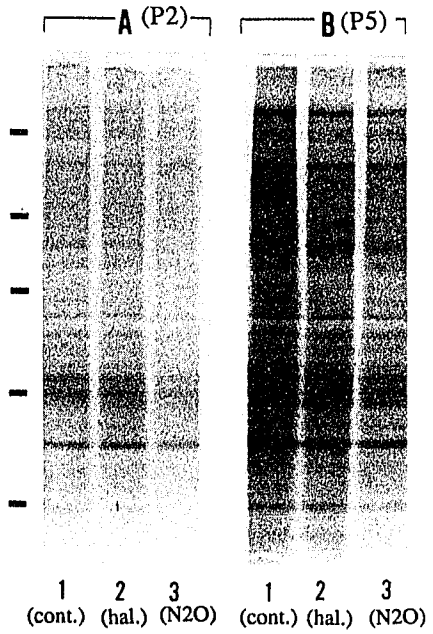

Fig. 2. Protein composition of growth cone particle (GCP). (*A*) P2 = GCP proteins isolated from P2 rat forebrain; (*B*) P5 = GCP proteins from P5; lane 1 in *A* or *B*, control group (cont.); lane 2, 1% halothane group (hal.); lane 3, 75% N_2O group. The standard molecular masses are also indicated on the left: from top to bottom, 200 kDa(myosin), 116 kDa(b-galactosidase), 97 kDa(phosphorylase b), 66 kDa(bovine serum albumin), and 45 kDa (ovalbumin). The acrylamide concentration of the gels was 10%.

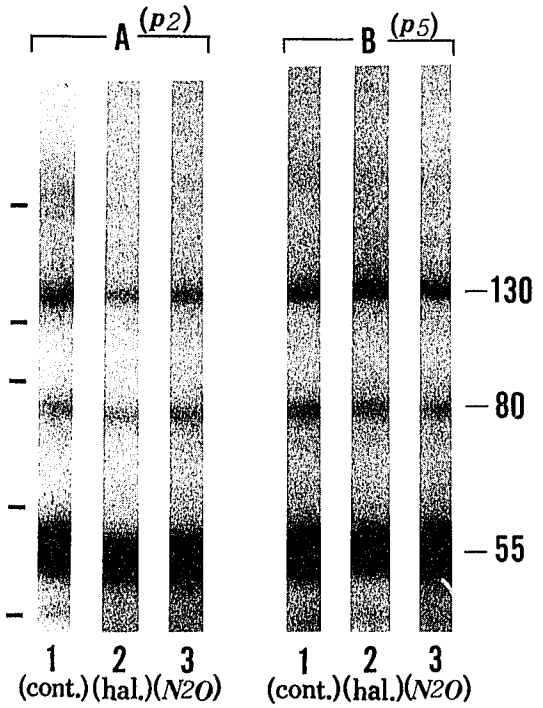

Fig. 3. Tyrosine phosphorylation of growth cone particle (GCP) protein. (*A*) *p2* = GCP proteins isolated from P2 rat forebrain; (*B*) *p5* = GCP proteins from P5; lane 1 in *A* or *B*, control group (cont.); lane 2, 1% halothane group (hal.); lane 3, 75% N_2O group. The molecular masses (kDa) are indicated on the right. The standard molecular masses are also indicated on the left: from top to bottom, 200 kDa(myosin), 116 kDa(b-galactosidase), 97 kDa(phosphorylase b), 66 kDa(bovine serum albumin), and 45 kDa(ovalbumin). The acrylamide concentration of the gels was 10%.

diograph appeared to have some alteration in density after exposure to the anesthetics, no statistical significance was obtained by densitometric quantification for other major bands. Direct assay of PKC activity using a synthesized specific substrate also indicated that PKC-activities in growth cone particles isolated from the anesthetic exposed groups (0.75, 1.0% halothane or 50, 75% nitrous oxide) were significantly lower than the corresponding values from the control group, at either 24 or 48 h after the exposure. However, 0.5% halothane and 25% nitrous oxide had no significant effect on PKC activity in growth cone particles (fig. 5).

Discussion

Neuroteratogenicity of Anesthetics

The teratogenicity of several anesthetics has been shown experimentally, although the methods of exposure of animals to anesthetics were not identical. Rodier *et al.* reported that even a brief exposure to 75% N_2O during the perinatal period had significant morphologic influences on the development of the nervous system and cell proliferation in the developing brain.[27] Quimby *et al.*[1] and Chang *et al.*[28] described that even a low concentration of anesthetics, such as 10 ppm halothane, had teratogenic effects on synapse formation in the brain, if chronically exposed during early life. Behavioral experiments also showed that several anesthetics, such as halothane or nitrous oxide, had detrimental effects on development without macro teratogenicity. Quimby *et al.*[2] and Smith *et al.*[3] described the behavioral effects of exposure to halothane during early development in the rat. Chalon *et al.*[4] reported that exposure to halothane or enflurane during the embryonal period affected learning functions in mice in later life. The exposure to N_2O was also demonstrated to be influential on neurologic development and behavior after birth if exposed during late gestation or during pregnancy.[5–7] These morphologic or behav-

Table 1. Relative Intensity of ^{32}P Incorporation of Each Major Tyrosine-phosphoprotein in GCP

Age / Anesthetics	Molecular Weight in SDS–PAGE (kDa) 55	80	130
P2			
Control	57.03 ± 3.71	7.72 ± 1.32	14.53 ± 2.13
N_2O	54.12 ± 2.39	9.75 ± 0.98	12.62 ± 1.31
Halothane	55.80 ± 5.48	9.20 ± 1.33	12.23 ± 1.63
P3			
Control	59.25 ± 5.00	7.82 ± 1.27	15.32 ± 1.31
N_2O	56.28 ± 5.04	10.18 ± 1.37	14.38 ± 0.98
Halothane	54.38 ± 4.84	9.47 ± 1.24	14.60 ± 1.33
P4			
Control	55.62 ± 3.77	8.32 ± 1.50	20.30 ± 1.25
N_2O	54.85 ± 7.04	7.55 ± 1.47	18.52 ± 1.72
Halothane	51.33 ± 5.37	8.27 ± 1.57	19.80 ± 1.42
P5			
Control	58.28 ± 3.11	8.40 ± 0.92	20.80 ± 1.91
N_2O	58.65 ± 5.44	7.90 ± 1.24	22.35 ± 2.05
Halothane	57.83 ± 3.25	7.88 ± 1.00	21.45 ± 1.46

After electrophoresis and autoradiography, each lane in the autoradiogram was scanned with a laser densitometer. The comparative intensity of each phosphoprotein band (percent to the total density of each lane) was calculated using a Basic program on a Hewlett-Packard computer. The molecular weight in SDS–PAGE of three major phosphoproteins are indicated in the top line. Values are expressed as means ± SEM (four independent experiments).

GCP = growth cone particle; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis; N_2O = 75% nitrous oxide; halothane = 1% halothane.

No statistical significance was observed between the value in the anesthetics-exposed group and that in the control group.

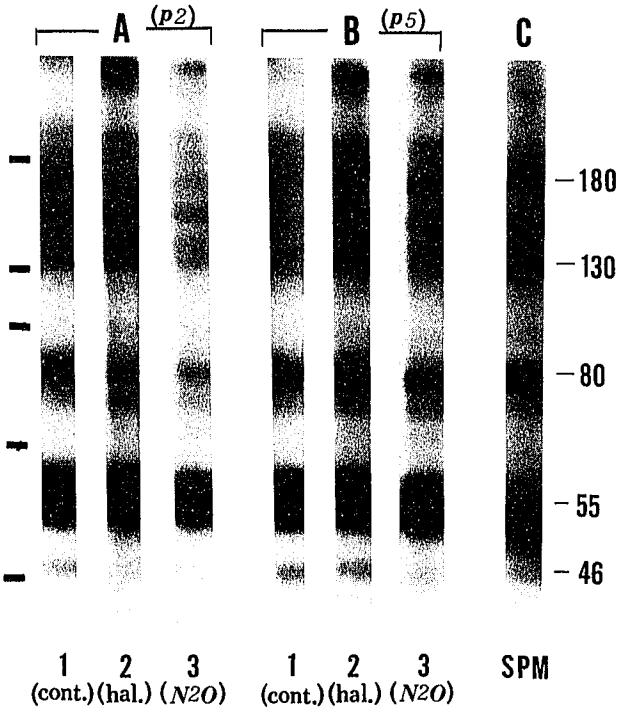

Fig. 4. Ca^{2+}-dependent phosphorylation of growth cone particle (GCP) protein. (*A*) GCP proteins isolated from *p2* rat forebrain; (*B*) GCP proteins from *p5*; (*C*) synaptosome (SPM) proteins isolated from adult rat forebrain. Lane 1 in *A* and *B* gels, control group (cont.); lane 2, 1% halothane group (hal.); lane 3, 75% N_2O group. The acrylamide concentration of the gels was 10%.

ioral abnormalities were apparently derived from some biochemical alteration in the developing brain, although the alterations were not necessarily identical in each experimental model. Until now, however, no biochemical information has been available to explain the underlying molecular mechanism of the effect of anesthetics on the developing brain.

Characteristics of Growth Cones in Developing Nervous System

Growth cones, the tips of growing neurites, are thought to play a crucial role in the establishment of the neuronal network.[11,12] This structure moves actively in the developing or regenerating nervous system, and guides neurite extension to construct the correct neural circuit. External signals from the environment, such as adhesion molecules, growth factors, or neurotransmitters, influence the movement of the growth cone, and probably provide the growth cones with information to find the correct path.[29] To receive and transduce these external signals, growth cones are equipped with receptors on the cytoplasmic membrane and with a system for intracellular signal transduction.[30] For example, we have reported that acetylcholine receptors are expressed and enriched in growth cone membranes with specific pharmacologic characteristics.[19] Recently, Igarashi *et al.* reported that the growth cone in a cultured neural tip collapsed *via* a G-protein linked signal transduction system when it came into contact with a myeline-derived substance.[31] With such characteristics, it may be that growth cones are the most sensitive site for drugs that induce synaptic malformation in the developing brain. What should be noted is that even a transient and reversible effect on the growth cone may evoke a permanent alteration in the neuronal circuit in the brain by misguiding the neurite extension at critical point in development, or inducing growth cone collapse or abnormal synaptogenesis. To clarify the molecular mechanism of events occurring in the developing neuron, the biochemical study of the growth cone could prove informative.

Protein Composition of Growth Cone After Exposure to Anesthetics

The experimental conditions that we adopted for exposure of the animals to anesthetics were comparable to previous reports that indicated neuroteratogenicity of anesthetics without macroteratogenicity.[8,9] In our experiments, we did not observe any detrimental influence on body weight or brain weight after exposure to the anesthetics, indicating that anesthetics had no macroteratogenic (gross malformation) effect. Comparison of the protein composition of growth cone particles among the groups also demonstrated that the major proteins were not influenced by exposure to the anesthetics, although minor proteins, which were difficult to examine with SDS-PAGE, were possibly influenced by the exposure. These observations indicated that the processes of protein synthesis, such as transcription, translation, and processing, and the transportation of the synthesized proteins are not significantly affected by exposure to the anesthetics.

Protein Phosphorylation in Growth Cone After Exposure to Anesthetics

Several growth cone proteins are reported to be phosphorylated by the intrinsic protein kinases, such as cAMP-dependent kinase, Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and tyrosine kinase.[17,18,20–22] These protein kinases were shown to be

Table 2. Relative Intensity of ^{32}P Incorporation of Each Major Ca^{2+}-dependent Phosphoprotein in GCP

Age / Anesthetics	Molecular Weight in SDS–PAGE (kDa)				
	46	55	80	130	180
P2					
Control	9.25 ± 0.19	48.43 ± 0.35	16.60 ± 0.46	8.77 ± 0.36	7.32 ± 0.32
N_2O	1.98 ± 0.13*	47.50 ± 0.75	5.39 ± 0.44*	9.68 ± 0.81	7.43 ± 0.39
Halothane	3.72 ± 0.32*	47.75 ± 0.69	6.52 ± 0.23*	11.30 ± 0.51	7.52 ± 0.27
P3					
Control	9.02 ± 0.23	49.20 ± 0.84	13.12 ± 0.99	8.72 ± 0.49	9.42 ± 0.27
N_2O	2.65 ± 0.22*	46.95 ± 1.29	6.47 ± 0.38*	11.10 ± 0.54	9.13 ± 0.72
Halothane	4.02 ± 0.28*	48.08 ± 1.52	7.44 ± 0.63*	10.68 ± 0.31	10.02 ± 0.50
P4					
Control	9.07 ± 0.58	48.00 ± 0.49	11.27 ± 0.76	8.94 ± 1.13	8.88 ± 0.64
N_2O	3.22 ± 0.21*	47.58 ± 0.90	6.99 ± 0.65†	9.14 ± 0.94	9.85 ± 0.35
Halothane	5.43 ± 0.26*	46.02 ± 1.04	7.80 ± 0.56†	10.39 ± 1.04	9.30 ± 0.29
P5					
Control	8.01 ± 0.15	48.50 ± 0.22	11.40 ± 0.33	7.94 ± 0.57	10.97 ± 0.70
N_2O	5.30 ± 0.29*	47.03 ± 0.46	8.50 ± 0.44*	8.26 ± 0.66	11.35 ± 0.93
Halothane	6.99 ± 0.44	46.60 ± 0.71	9.34 ± 0.36†	8.81 ± 0.71	12.97 ± 0.41

After electrophoresis and autoradiography, each lane in the autoradiogram was scanned with a laser densitometer. The comparative intensity of each phosphoprotein band (percent to the total density of each lane) was calculated using a Basic program on a Hewlett-Packard computer. The molecular weight in SDS–PAGE of five major phosphoproteins are indicated in the top line. Values are expressed as means ± SEM (four independent experiments).

GCP = growth cone particle; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis; N_2O = 75% nitrous oxide; halothane = 1% halothane.

The value in the anesthetics-exposed group was statistically compared to the corresponding control value:

* $P < 0.01$.

† $P < 0.05$.

key enzymes in an intracellular signal transduction system. Tyrosine phosphorylation is suggested to function at receptor sites for several kinds of growth factors, or at the cytoskeleton regulatory sites in the nervous system.[32,33] The calcium-dependent phosphorylation is thought to regulate the receptor proteins, ion channel proteins, and several kinds of functional proteins, such as GAP-43 and synapsin I.[34] In our experiment, the tyrosine phosphorylation in growth cone particles was not markedly affected by exposure to the anesthetics. In contrast, autoradiographic or densitometric examinations clearly demonstrated that the pattern of Ca^{2+}-dependently phosphorylated proteins was significantly influenced by the exposure. In particular, phosphorylation of the 43-kDa and 80-kDa proteins was most strikingly affected. These proteins were established as being a specific substrate for protein kinase C, and both of them are reported to play a special role in the developing nervous system.[26] The 43-kDa protein, which is now known as GAP (growth associated protein)-43 or as B-50, is considered to function as a regulator of calcium concentration in the presynaptic terminal, probably by binding to calmodulin.[35–37] The phosphorylation of this protein is prominent in the growth cone when compared with the adult synapse.[21,26] It is also thought to be important in memory formation in the adult brain, because the phosphorylation of this protein is augmented during long-term potentiation in the hippocampus.[35,38] The 80-kDa protein is known as the MARKS (mylistoylated alanine rich kinase substrate) protein because of its biochemical characteristics.[39] The phosphorylation of this protein is also reported to be related to the synaptic plasticity; however, the precise function of this protein has not been elucidated.[40]

The results of gel-autoradiography indicated that several other minor phosphoproteins, which we did not intensively examine in this study, were also affected to some extent after exposure to anesthetics. Because several proteins are known to be phosphorylated by several kind of kinase systems, it is possible that these proteins are partially phosphorylated by PKC. Moreover, some kinases are reported to be phosphorylated by PKC, thus altering their functions. Therefore, phosphoproteins that are phosphorylated by other kinase systems may also be influenced, to some extent, by the modification of PKC activity. Although alterations in phosphoproteins, other than 46kDa or 80kDa, may indicate their role in the

mechanism involved in the neural effect of anesthetics in developing brain, further basic biochemical investigation is necessary to evaluate this phenomenon from a pharmacologic viewpoint.

Protein Kinase C in Growth Cone

The decrease in ^{32}P incorporation into 43- and 80-kDa proteins indicated that the phosphorylation by PKC was disrupted after exposure to the anesthetics. The direct measurement of PKC activity using an exogenous substrate peptide confirm the autoradiography results, although the statistical significance of the results obtained by the two different assay methods are not in complete agreement at P4 or P5. This disagreement in statistical significance is most probably derived from the difference in the assay systems; for example, the degree of sensitivity, specificity, or reproducibility. However, the endogenous substrate proteins, which were not the main kinase substrate in the direct assay of PKC activity (in which excessively added exogenous substrate peptide were mainly used as kinase substrate), were possibly affected by exposure to the anesthetics. In the PKC direct assay experiment, a dose–response relationship between concentration of exposed anesthetics and kinase inhibition was observed to a certain degree. Therefore, this alteration in PKC activity may be derived from some pharmacologic action of the anesthetics. PKC is a key enzyme functioning in cell-surface signal transduction in various cellular processes.[41] This enzyme is most abundant in the brain, and is implicated in modulating neuronal functions, such as neurotransmitter release, ion channel conductance, and receptor sensitivity in the adult brain.[42] There is also increasing evidence that PKC is involved in neuronal differentiation and plasticity. We have already reported that PKC is expressed in growth cones with a unique subtype distribution, and have implied a distinctive function for this enzyme in signal transduction in growth cones.[43] Local anesthetics, such as tetracaine and procaine, are known to inhibit the activity of PKC.[44] In contrast, Tsuchiya *et al.* reported that the activity of purified protein kinase C was augmented by the presence of volatile anesthetics, probably through perturbation of the lipid bilayer.[45] It is also well known that the activity of PKC is downregulated after activation *via* an autophosphorylation mechanism.[46] Although it is possible that the purified enzyme and the *in situ* enzyme react differently with the anesthetics, the direct action of anesthetics on PKC is possibly related to the long-term decrease in the kinase activity, which we observed in growth cone particles after exposure to the anesthetics. Considering the role of growth cones in synaptogenesis and development of the neuronal network, a long-term effect on PKC activity in growth cones by anesthetics may interfere with signal transduction in the growth cone, and, thereafter, induce synaptic malformations, as demonstrated previously by morphologic or behavioral experiments.

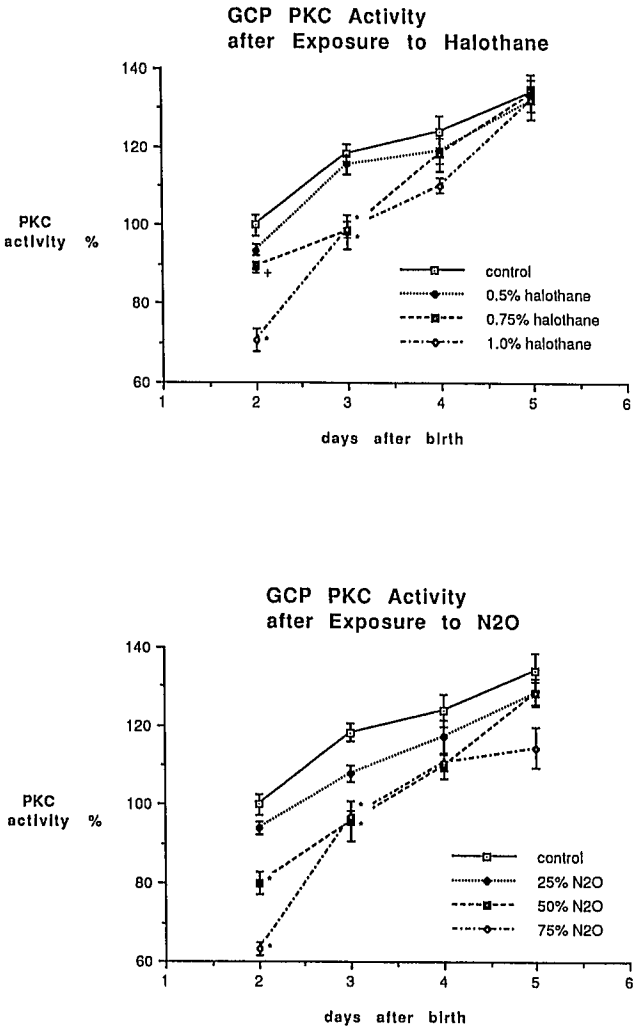

Fig. 5. Protein kinase C (PKC) activities in growth cone particle (GCP). The PKC activities in each sample are expressed as the percentages of the value in control GCP at P2. Data are given as the mean ± SEM from four independent experiments. The values in anesthetic-exposed groups were statistically compared with the value in control group at corresponding date. *$P < 0.01$; †$P < 0.05$.

Molecular Mechanism of Anesthetic Action on Developing Nervous System

Nitrous oxide and halothane, which we examined in this study, are thought to act as anesthetics through different molecular mechanisms. Although the precise mechanism of anesthetic action is not fully understood even now, volatile anesthetics, such as halothane, enflurane, isoflurane, and sevoflurane, are thought to exert their action by altering the conductance of the ion channel in the lipid bilayer.[47,48] In contrast, nitrous oxide is thought to act by modulating the inhibitory neurotransmission, or by interaction with the cGMP-nitric oxide system.[49-51] Although the results for halothane and those for N_2O, which we observed in this study, qualitatively resemble each other, the action on protein kinase C of each anesthetic may be derived from a distinct underlying mechanism. After the behavioral observations, Rodier *et al.* and Herman *et al.* showed that halothane and nitrous oxide, which produce very different degrees of anesthesia, act similarly as neuroteratogens if applied during development, and they suggested that the neuroteratogenicity of inhalants is independent of the level of anesthesia produced.[8-10]

To clarify the precise action of anesthetics on the developing nervous system, further biochemical and physiologic studies on the molecular mechanism of anesthetic action and brain development are indispensable. Any further information about the neurochemical characteristics of brain development, especially that of growth cones, may be useful in investigations of the pharmacology or toxicology of drugs, including anesthetics, in the developing nervous system.

In summary, we have observed that the exposure to general anesthetics have significant influence on biochemical events in the developing neural tips. This phenomenon possibly causes further morphologic or behavioral abnormalities in later life.

References

1. Quimby KL, Ashkenase LJ, Bowman RE: Enduring learning deficits and cerebral synaptic malformation from exposure to 10 parts of halothane per million. Nature 185:625–627, 1974

2. Quimby KL, Katz J, Bowman RE: Behavioral consequences in rats from chronic exposure to 10 ppm halothane during early development. Anesth Analg 54:628–632, 1975

3. Smith RF, Bowman RE, Katz J: Behavioral effects of exposure to halothane during early development in the rat. ANESTHESIOLOGY 49:319–323, 1978

4. Chalon J, Tang CK, Ramanathan S, Eisner M, Katz R, Turndorf H: Exposure to halothane and enflurane affects learning function of murine progeny. Anesth Analg 60:794–797, 1981

5. Tassinari MS, Mullenix PJ, Moore PA: The effects of nitrous oxide after exposure during middle and late gestation. Toxicol Ind Health 2:261–271, 1986

6. Mullenix PJ, Moore PA, Tassinari MS: Behavioral toxicity of nitrous oxide in rats following prenatal exposure. Toxicol Ind Health 2:273–287, 1986

7. Rice SA: Effect of prenatal N_2O exposure on startle reflex reactivity. Teratology 42:373–381, 1990

8. Koeter HBWM, Rodier PM: Behavioral effects in mice exposed to nitrous oxide or halothane: Prenatal vs. postnatal exposure. Neurobehav Toxicol Teratol 8:189–194, 1986

9. Rodier PM, Koeter HBWM: General activity from weaning to maturity in mice exposed to halothane or nitrous oxide. Neurobehav Toxicol Teratol 8:195–199, 1986

10. Rodier PM: Inhalant anesthetics as neuroteratogens. Ann N Y Acad Sci 477:42–48, 1986

11. Lockerbie RO: The neuronal growth cone: A review of its locomotory, navigational and target recognition capabilities. Neuroscience 20:719–729, 1987

12. Van Hoof COM, Oestreicher AB, De Graan PNE, Gispen WH: Role of the growth cone in neuronal differentiation. Mol Neurobiol 3:101–133, 1989

13. Pfenninger KH, Ellis L, Johnson MP, Friedman LB, Somlo S: Nerve growth cones isolated from fetal rat brain: I. Subcellular fractionation and characterization. Cell 35:573–584, 1983

14. Gordon-Weeks RR, Lockerbie RO: Isolation and partial characterisation of neuronal growth cones from neonatal rat forebrain. Neuroscience 13:119–136, 1984

15. Igarashi M, Waki H, Hirota M, Hirabayashi Y, Obata K, Ando S: Differences in lipid composition between isolated growth cones from the forebrain and those from the brain stem in the fetal rat. Dev Brain Res 51:1–9, 1990

16. Saito S, Fujita T, Komiya Y, Igarashi M: Biochemical characterization of nerve growth cones isolated from both fetal and neonatal rat forebrains: The growth cone particle fraction mainly consists of axonal growth cones in both stages. Dev Brain Res 65:179–184, 1992

17. Igarashi M, Saito S, Komiya Y: Vinculin is one of the major endogenous substrates for intrinsic tyrosine kinases in neuronal growth cones isolated from fetal rat brain. Eur J Biochem 193:551–558, 1990

18. Igarashi M, Komiya Y: Tyrosine phosphorylation and immunodetection of viculin in growth cone particle fraction (GCP) and in GCP-cyoskeletal subfraction. J Neurosci Res 30:266–274, 1991

19. Saito S, Komiya Y, Igarashi M: Muscarinic acetylcholine receptors are expressed and enriched in growth cone membranes isolated from fetal and neonatal rat forebrain: Pharmacological demonstration and characterization. Neuroscience 45:735–745, 1991

20. Ellis L, Katz F, Pfenninger KH: Nerve growth cones from fetal rat brain: II. Cyclic adenosine 3',5'-monophosphate(cAMP)-binding proteins and cAMP-dependent phosphorylation. J Neurosci 5:1393–1401, 1985

21. Katz F, Ellis L, Pfenninger KH: Nerve growth cones from fetal rat brain: III. Calcium-dependent protein phosphorylation. J Neurosci 5:1402–1411, 1985

22. Lockerbie RO, Edde B, Prochiantz A: Cyclic AMP-dependent protein phosphorylation in isolated neuronal growth cones from developing rat forebrain. J Neurochem 52:786–796, 1989

23. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein dye binding. Anal Biochem 72: 248–254, 1976

24. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970

25. Wada S, Yasumoto Y, Kosano H, Kugai N, Nagata N: The effect of PGF_{2a} on parathyroid hormone-stimulated cyclic AMP production in mouse osteoblastic cell, $MC3T3E_1$. Biochem Biophys Acta 1074: 182–188, 1991

26. Nelson RB, Linden DJ, Hyman C, Pfenninger KH, Rottenberg A: The two major phosphoproteins in growth cones are probably identical to two protein kinase C substrates correlated with persistence of long-term potentiation. J Neurosci 9:381–389, 1989

27. Rodier PM, Aschner M, Lewis LS, Koeter HBWM: Cell proliferation in developing brain after brief exposure to nitrous oxide or halothane. ANESTHESIOLOGY 64:680–687, 1986

28. Chang LW, Dudley AW, Katz J: Pathological changes in the nervous system following in utero exposure to halothane. Environ Res 11:40–51, 1976

29. Strittmatter SM, Fishman MC: The neuronal growth cone as a specialized transduction system. Bioessays 13:127–134, 1991

30. Lockerbie RO: Biochemical pharmacology of isolated neuronal growth cones: Implications for synaptogenesis. Brain Res Rev 15: 145–165, 1990

31. Igarashi M, Stritmatter SM, Vartanian T, Fishman MC: Mediation by G proteins of signals that cause collapse of growth cones. Science 259:77–79, 1993

32. Yarden Y: Growth factor receptor tyrosine kinases. Ann Rev Biochem 57:443–478, 1988

33. Wagner KR, Mei L, Huganir RL: Protein tyrosine kinases and phosphatases in the nervous system. Curr Opin Neurobiol 1:65–73, 1991

34. Schulman H: Serine/threonine kinases in the nervous system. Curr Opin Neurobiol 1:43–52, 1991

35. De Graan PNE, Schrama LH, Heemskerk FMJ, Dekker LV, Gispen WH: The role of protein kinase C substrate B-50 (GAP-43) in neurotransmitter release and long-term potentiation, Exicitatory Amino Acids and Neuronal Plasticity. Edited by Ben-Ari Y. New York, Plenum, 1990, pp 347–358

36. Sheu FS, Kasamatsu T, Rottenberg A: Protein kinase C activity and substrate (F1/GAP-43) phosphorylation in developing cat visual cortex. Brain Res 524:144–148, 1990

37. Meiri KF, Bickerstaff LE, Schwob JE: Monoclonal antibodies show that kinase C phosphorylation of GAP-43 during axonogenesis is both spatially and temporally restricted in vivo. J Cell Biol 112: 991–1005, 1991

38. Daigen A, Akiyama K, Otsuki S: Long-lasting change in the membrane-associated protein kinase C activity in the hippocampal kindled rat. Brain Res 545:131–136, 1991

39. Stumpo DJ, Graff JM, Albert KA, Greengard P, Blackshear PJ: Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: A major cellular substrate for protein kinase C. Proc Natl Acad Sci U S A 86:4012–4016, 1989

40. Girard PR, Kuo JF: Protein kinase C and its 80-kilodalton substrate protein in neuroblastoma cell neurite outgrowth. J Neurochem 54:300–306, 1990

41. Nishizuka Y: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665, 1988

42. Huganir RL, Greengard P: Regulation of neurotransmitter receptor desensitization by protein phosphorylation. Neuron 5:555–567, 1990

43. Igarashi M, Komiya Y: Subtypes of protein kinase C in isolated nerve growth cones: Only type II is associated with the membrane skeleton from growth cones. Biochem Biophys Res Commun 178: 751–757, 1991

44. Trudell JR, Costa AK: Inhibition of protein kinase C by local anesthetics. Ann N Y Acad Sci 625:743–746, 1990

45. Tsuchiya M, Tomoda M, Ueda W, Hirakawa M: Halothane enhances the phosphorylation of H1 histone and rat brain cyoplasmic proteins by protein kinase C. Life Sci 46:819–825, 1990

46. Ohno S, Konno Y, Akita Y, Yano A, Suzuki K: A point mutation at the putative ATP-binding site of protein kinase C α abolishes the kinase activity and renders it down-regulation-insensitive. J Biol Chem 265:6296–6300, 1990

47. Krnjevic K: Cellular mechanisms of anesthesia. Ann N Y Acad Sci 625:1–16, 1990

48. Drenger B, Heitmiller ES, Quigg M, Blanck TJ: Depression of calcium channel blocker binding to rat brain membranes by halothane. Anesth Analg 74:758–761, 1992

49. Chapman CR, Benedetti C: Nitrous oxide effects on cerebral evoked potential to pain. ANESTHESIOLOGY 51:135–138, 1979

50. Yang JC, Clark WC, Ngai SH: Antagonism of nitrous oxide analgesia by naloxone in man. ANESTHESIOLOGY 52:414–417, 1980

51. Huang C, Liu G, Hsiao CF, Huang R: Electrophysiology of ethanol, nitrous oxide, and barbiturate on presumed subtypes of cellular granule cells. Ann N Y Acad Sci 625:264–268, 1990