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Differential effects of isoflurane, halothane, and ketamine on the regional methionine-enkephalin-like immunoreactivity in the mouse brain

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Abstract: The widely used measurement index for anesthetic potency, minimum alveolar concentration (MAC), is hypothesized to be the sum of the effects on multiple neural systems whose contribution to anesthesia differs depending on the agents used. The present study, which compared the effects of halothane, isoflurane, and ketamine, at equipotent level of anesthesia, on the methionine-enkephalinergic neurons in 9 brain regions, showed a significant difference in the methionine-enkephalin-like immunoreactivity (Met-ENK-like IR) among the anesthetics in each region. The order of the Met-ENK-like IR was: halothane > ketamine > isoflurane in the caudatus putamen; halothane > isoflurane ≅ ketamine in the nucleus accumbens and the ventral pallidum; halothane ≅ isoflurane > ketamine in the globus pallidus, the nucleus dorsomedialis hypothalami, and the nucleus ventromedialis hypothalami; and halothane > isoflurane > ketamine in the arcuate nucleus, the periaqueductal gray, and the nucleus reticularis parvocellularis. These findings indicate that these three anesthetics affect the methionine-enkephalinergic neurons in the motor and pain controlling pathways in different fashions.

Introduction

The ratio of minimum alveolar concentration (MAC) assessed by tail-clamping to the ED_{50} for loss of the righting reflex has been shown to vary significantly for different anesthetics [1–3]. In those studies, it was hypothesized that the response at MAC was the sum of the effects on motor controlling and pain transferring systems, and the contributions of these two neural systems were different from agent to agent. This hypothesis suggests that anesthetics change the activity of the neural pathways which belong to the motor controlling and pain transferring systems, in a balance varying for different anesthetics.

Methionine-enkephalin (Met-ENK) neurons, which are distributed widely in the central nervous system [4–6], are known to associate prominently with motor and pain controlling pathways [7–9]. A series of studies concerning the relationship between anesthetics and endogenous opiates have been reported [10–15]. While the results of these studies are controversial, the endogenous opiates may participate in anesthesia, especially in the component of analgesia. In the present study, using immunohistochemistry we investigated whether different anesthetics affect Met-ENK in these neural pathways in different ways. We chose halothane, isoflurane, and ketamine for the comparison because of their wide variety of the analgesic potency and the effects on motor system, such as muscle relaxation, and the incidence of involuntary movements [16–20].

Materials and methods

Male ddN mice for the experiments were inbred in the Animal Laboratory of Kagawa Medical School under a 12-h light-dark cycle (lights on at 6:00 a.m., off at 6:00 p.m.) at 24°C with food and water freely available. The 19 genetic markers of the 9 mice selected at random were investigated with a Titan III electrophoretic system (Helene) and by isoelectric focusing (IEF). The examination of genetic profile confirmed the genetic

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homogeneity of the nine mice selected at random (by Dr. Katoh, Central Institute for Experimental Animals).

Preliminary experiments for the determination of equipotent doses of volatile anesthetics

Twenty male mice of ddN strain aged 9 ~ 11 weeks were separated into four groups, each of which received either 1.5% isoflurane, 1.7% isoflurane, 1.3% halothane, or 1.5% halothane. Air containing each concentration of anesthetics was administered into 1-l and 30-ml plastic bags through a vaporizer at a flow rate of 2 l/min. Concentrations of anesthetics were continuously monitored using an infrared anesthetic gas analyzer (Normac, Datex, Helsinki, Finland). Thirty min after gas flow was established, the mice were transferred into the 1-l bag and anesthetized. A few minutes later, they were removed from the bag and the heads were placed into the 30-ml bag for the tail-clamping test. Fifteen min after the induction of the anesthesia, the base of the tail was clamped with a hemostat (15 cm) for 10 s three times with a 30-s interval. When the mouse showed a purposeful movement of the head or legs in three trials, the response was considered to be positive. All mice in the groups which received 1.5% isoflurane and 1.3% halothane showed positive responses. In the other two groups, no positive response was observed.

Anesthesia for perfusion fixation

Fifteen male mice of ddN strain, aged 8 ~ 11 weeks, were separated into three groups: isoflurane, halothane, and ketamine. Administration of anesthetics and perfusion fixation were performed between 2:00 p.m. and 5:00 p.m.

Isoflurane and halothane groups. The same anesthesia apparatus was used as in the preliminary experiments to administer 1.7% isoflurane or 1.5% halothane to these two groups. They received tracheotomies and were intubated with intratracheal cannula. Artificial ventilation was started with above concentrations of anesthetics in air using a ventilator (AIKA EVM-50A, Japan). After i.p. administration of 0.04 mg of pancuronium bromide and 0.7 ml of 0.7% $NaHCO_3$ in saline, anesthesia was continued for 60 min. The esophageal temperature was monitored and maintained at 37°–37.8°C.

Ketamine group. In the mice, sufficient doses for the loss of righting reflex are less than the doses which abolish the response to the tail-clamping. The mice of this group received 167 mg·kg^{-1}/0.1 ml ketamine hydrochloride intraperitoneally. After the loss of righting reflex, the mice received tracheotomies using a local anesthetic (0.05 ml of 1% lidocaine). The mice were artificially ventilated with air. The tail-clamping test was performed 25 min after induction according to the same protocol as in the preliminary experiments for the determination of the equipotent doses of volatile anesthetics. When the animals showed a positive response, 1 mg ketamine was added. The tail-clamp test and the injection of ketamine were repeated with 15-min intervals until the mouse lost positive responses; then the same doses of pancuronium bromide and $NaHCO_3$, as in the halothane and isoflurane groups, were injected intraperitoneally. Artificial ventilation was continued for 60 min from the first injection of ketamine. The esophageal temperature was monitored with a probe inserted into the esophagus at a depth of 30 mm from the incisor teeth and was maintained at 37°–38°C.

Tissue preparation and immunohistochemical staining

Sixty min after the induction of anesthesia, the mice of all groups underwent arterial blood gas examinations and were perfused via the left cardiac ventricle with 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4). The brain was extracted and postfixed in the same solution for an additional 24 h and immersed in 0.1% phosphate buffer containing 20% sucrose for 48 h at 4°C. Then the brains, from the medulla oblongata to the rostral end of the caudatus putamen, were cut in 20-μm coronal sections with a cryostat and were divided in turns into 5 groups. One group was incubated in an antiserum against Met-ENK (Incstar, Stillwater, Minnesota, USA) for 7 days at 4°C, then processed using the avidin biotin complex technique at 26°C according to the method of Kawata et al. [21]. The antiserum was raised in rabbit against methionine-enkephalin coupled to bovine thyroglobulin with glutaraldehyde. In the avidin biotin complex technique, we used the biotinylated affinity-purified goat anti-rabbit IgG. Another group of sections received Nissl staining for anatomical mapping.

Image analysis

The Met-ENK-like immunoreactivity (Met-ENK-like IR) of target regions was evaluated using an image analysis system (Zeiss IBAS-1,2, Germany) according to Zoli et al. [22]. After the calibration of light power and magnification, we selected the region of interest using a cursor which appeared along with the image on a video monitor. The image analysis system determines the average gray value (AGV) representing the optical lucency of the region. To investigate the reproducibility of the densitometric recordings and calibration, we

measured the AGV of a target region ten times for successive ten calibrations. To cancel out the nonspecific staining, we calculated the specific AGV (sAGV) which is equal to the AGV of the corpus callosum minus the AGV of the target nucleus in the same group of sections. No specific staining was observed in the corpus callosum in the previous reports [4]. We used the corpus callosum as the nonspecifically stained region. Each measurement was carried out three times. The statistical analyses used were one-way analysis of variance and the Mann-Whitney U-test.

Immunoabsorption test

The specificity of immunohistochemical localization was determined according to Williams and Dockray [5]. We preincubated the antiserum at 4°C for 24h with Met-ENK and Leucine-ENK at 10 and 100nmol/ml, respectively. After the preincubation, the immunohistochemical staining and the image analysis were carried out as described above.

Results

The total doses of ketamine were 6~7mg per animal. The arterial blood gas data were normocapnic and not metabolically acidotic for all mice.

Image analysis showed good reproducibility in AGV evaluation, since the coefficient of variation of the 100 times (on each of ten successive calibrations) was within 2%.

The microphotographs of Met-ENK-like-IR are shown in Figs. 1–3 and their sAGV in Table 1. The sAGVs of 4 regions, caudatus putamen (cp), arcuate nucleus (AN), periaqueductal gray (PAG), and nucleus reticularis parvocellularis (nrp) were significantly different among the three groups. In the other five regions, nucleus accumbens (N.acc), ventral pallidum (vp), globus pallidus (gp), nucleus dorsomedialis hypothalami (ndm), and nucleus ventromedialis hypothalami (nvm), the sAGV of one group showed significant difference from the other two groups. Furthermore, the order of magnitude of the Met-ENK-like IR in three groups was not the same among nine regions: halothane > ketamine > isoflurane in the cp; halothane > isoflurane ≅ ketamine in the N.acc and vp; halothane ≅ isoflurane > ketamine in the gp, ndm, and nvm; and halothane > isoflurane > ketamine in the AN, PAG, and nrp.

Staining with the antiserum was abolished by preincubation of the antiserum with Met-ENK except in the gp which showed a low IR. Preincubation with Leucine-ENK had so little effect that sAGV of each region showed no significant difference from the control (data not shown).

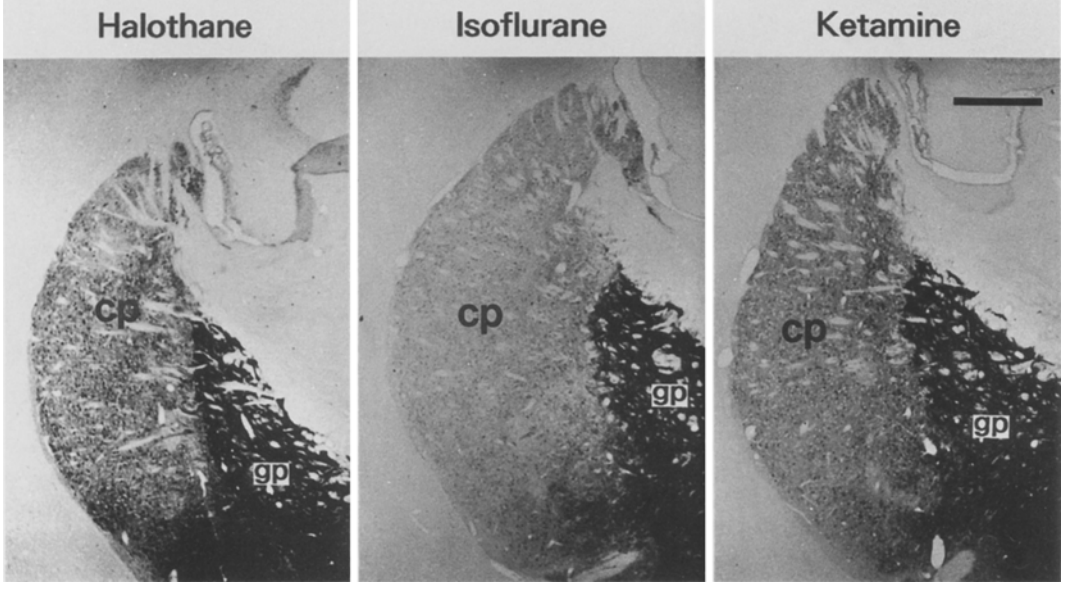

Fig. 1. Met-Enk-like immunoreactivity (IR) in the caudatus putamen (*cp*) and globus pallidus (*gp*) at the level of the coronal section 281 of the Sidman's atlas of the mouse brain [28]. Small scattered immunoreactive cells and fine fibers are seen in the cp, and the immunoreactivity is highest in the halothane group, moderate in the ketamine group, and lowest in the isoflurane group. Dense accumulation of immunoreactive fibers is seen in the gp. The IR in the ketamine group is lower than that of the other two groups, especially in the external part of the gp (Scale bar = 500 μm)

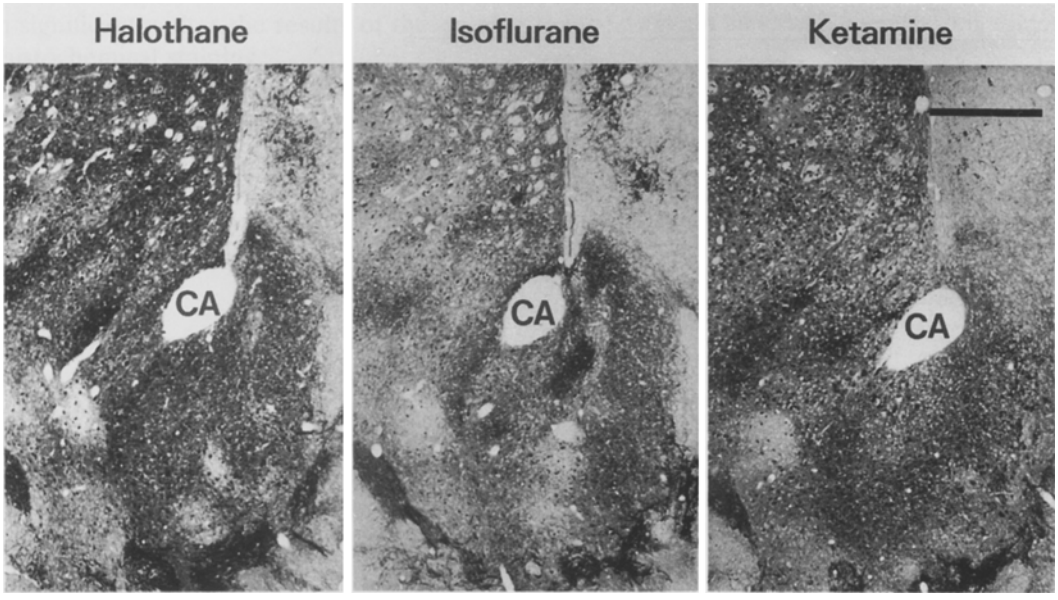

Fig. 2. Met-ENK-like IR in the nucleus accumbens (N.acc). Scattered immunoreactive cells and fibers surrounding the anterior commisure (*CA*) are seen. The halothane group shows a higher intensity of immunoreactivity than the other two groups (Scale bar = 500 μm)

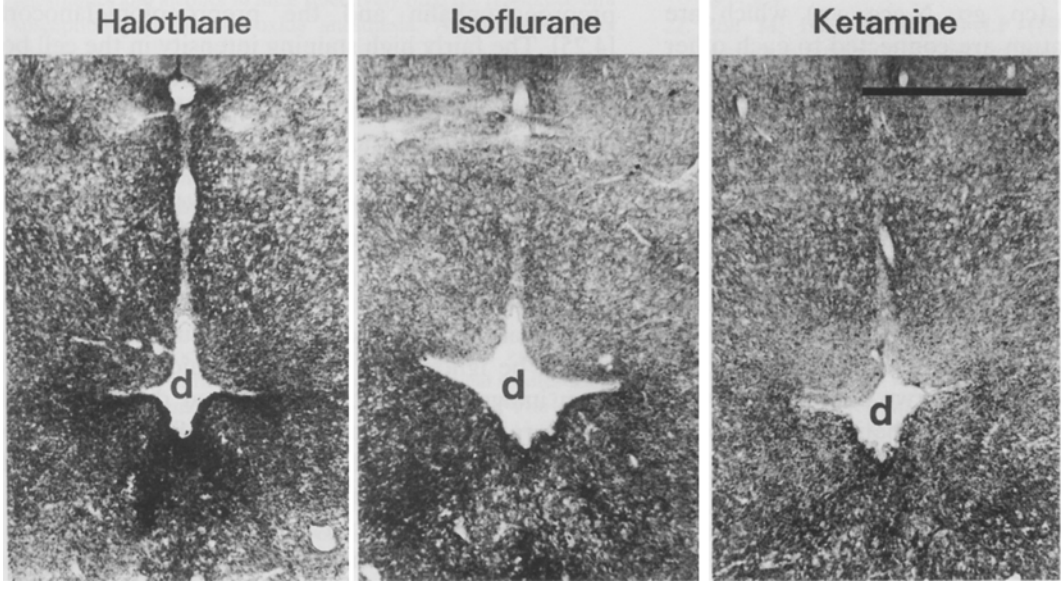

Fig. 3. Met-ENK-like IR in the periaqueductal gray matter. The varicosities surrounding the aqueductus cerebri (*d*) are seen (Scale bar = 500 μm)

Discussion

The present study clearly showed a significant difference between the anesthetic groups in the Met-ENK-like IR in each brain region after 1-h administration of equipotent levels of anesthesia assessed by the tail-clamping test (Table 1). The Met-ENK-like IR represents the content of the Met-ENK-like immunoreactive substrates. Therefore, the differential Met-ENK-like IR can be interpreted as the sum of the specific effects of anesthetics on several factors which determine the content of the substrates, that is, gene transcription, posttranslational processing, axonal transport, and release dynamics. Anesthetics could affect these neural functions both directly and indirectly via the afferent inputs to each brain region. The contribution of these

Table 1. The specific average gray values in nine brain regions

Brain regions	Specific average gray value (mean ± SD)		
	Halothane	Isoflurane	Ketamine
cp	88.9 ± 10.1 (44)*,†	51.3 ± 7.9 (49)	71.8 ± 8.1 (55)##
N.acc	119.5 ± 7.1 (45)*,†	102.2 ± 9.4 (38)	103.3 ± 9.3 (34)
vp	163.0 ± 4.8 (46)*,†	159.5 ± 3.7 (43)	157.8 ± 6.1 (43)
gp	181.8 ± 2.3 (45)†	184.3 ± 3.0 (44)#	173.1 ± 3.0 (40)
ndm	100.2 ± 12.4 (49)†	99.9 ± 12.8 (46)#	74.1 ± 9.8 (49)
nvm	87.0 ± 16.2 (49)†	79.9 ± 14.3 (46)#	61.4 ± 12.7 (49)
AN	80.3 ± 9.7 (49)a,†	56.0 ± 14.6 (46)#	38.5 ± 12.4 (49)
PAG	82.4 ± 11.0 (55)**,†	77.6 ± 11.2 (54)#	64.6 ± 11.8 (56)
nrp	95.8 ± 13.0 (42)a,†	84.3 ± 7.5 (41)#	71.1 ± 11.0 (42)

The values in parentheses indicate number of sections in the respective group.
cp, caudatus putamen; N.aac, nucleus accumbens; vp, ventral pallidum; gp, globus pallidus; ndm, nucleus dorsomedialis hypothalami; nvm, nucleus ventromedialis hypothalami; AN, arcuate nucleus; PAG, periaqueductal gray; nrp, nucleus reticularis parvocellularis.
*halothane > isoflurane $P < 0.01$, **halothane > isoflurane $P < 0.05$, †halothane > ketamine $P < 0.01$, #isoflurane > ketamine $P < 0.01$, ##ketamine > isoflurane $P < 0.01$.

factors to the differential Met-ENK-like IR would not be identical for each brain region because of the anatomical regional specificity such as the composition of the neural structures (cell bodies, axons, and terminals), the afferent connections, and the receptors which distribute within the region [23].

The four regions (cp, gp, N.acc, vp) which are comprised in the striatum are connected to each other to make two enkephalinergic neural tracts, the cp-gp tract and the N.acc-vp tract. In these two neural tracts, the IR of the three anesthetics groups showed different distributions. The order of IR in the cp, which contains the cell bodies of the cp-gp tract, is halothane > ketamine > isoflurane. In the terminal region of the cp-gp tract (gp), the ketamine group showed lower IR than the other two groups. The IR of the N.acc-vp tract was higher in the halothane group than the other two. These specific distributions indicated that the enkephalinergic neural activities in these two neural tracts were different among the three anesthetics. The specific effects of anesthetics on the motor controlling systems may be attributed, in part, to the differential neural activities in these two enkephalinergic neural tracts.

The PAG and the AN are the components of the descending pain controlling pathways. In these regions the IR of the three anesthetic groups showed significant differences. These findings indicate that the equipotent levels of these three anesthetics assessed by tail-clamping are associated with different levels of neural activity in these regions.

Immunohistochemical staining has advantages over the punched out method in terms of regional identification. We could evaluate the regions of the entire brain according to their anatomical boundary. In terms of quantification, an optimal staining condition is reported to have a linear relationship between sAGV and the content of the antigens [24]. Our method cannot evaluate the absolute amount of the antigen; however, the difference of the staining intensity can represent the differential content of the antigen. The immunoabsorption test revealed that the antiserum had high selectivity to the free Met-ENK. The Met-ENK also exists as an internal sequence of the two precursors; preproenkephalin and the preproopiomelanocortin [4,25]. The fairly high staining intensity in the cell bodies in the cp and N.acc, which are reported to contain no free Met-ENK in the rat [5], indicated that the antiserum may bind not only to free Met-ENK but also to these precursors.

The anesthesia level which we used in this study was just above the dose necessary to eliminate the response to the tail-clamping. At lower doses, the perfusion fixation cannot be performed without any additional anesthesia. At higher doses, the circulatory depression cannot be ignored. A number of mice were excluded from image analysis due to marked decreases in the heart rate and blood pressure. Some circulatory support, for example, the administration of catecholamines, is essential for the maintenance of deeper anesthesia. Both lighter and deeper levels of anesthesia require physiological changes which may affect neural activity in the central nervous system. Therefore we could not investigate how the Met-ENK-like IR changed in proportion to the depth of anesthesia.

The tissue preparation using the perfusion fixation technique has been widely used in the histochemical studies, due to better tissue fixation and immunohistochemical staining [26]. Histochemists have not paid enough attention to the essential anesthesia for the animals during the perfusion fixation, although Winters reported on the possible influence of the anesthetics on the neurophysiological experiment [27]. The results of the present study proved that the choice of anesthetics

can significantly alter the results of the ensuing immunohistochemical staining.

In summary, we observed the differential effects of halothane, isoflurane, and ketamine on the Met-ENK-like IR in nine brain regions. These results may indicate that the three anesthetics influence the enkephalinergic neural pathways in the motor and pain controlling systems differently. This suggests a multisite mechanism of anesthesia also.

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