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Effects of biogenic amines and intravenous anesthetics on the activity of rat locus coeruleus neurons in vitro

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Abstract: To examine the effects of biogenic amines and clinically relevant concentrations of intravenous anesthetics on neuronal activities, the authors analyzed both spontaneous and evoked activities of neurons in the nucleus locus coeruleus (LC) *in vitro* using a single unit recording technique. Spontaneous firing was observed in 37% (14/38) of LC neurons, and *N*-methyl-D-aspartate (NMDA, 50 μM), glutamate (250 μM), and carbachol (1–2 mM) elicited firing in 100% (38/38), 63% (12/19), and 58% (7/12) of silent LC neurons, respectively. Noradrenaline (50 μM) and serotonin (5-HT) (1–5 μM) suppressed spontaneous and drug-induced activities in 47% (15/32) and 23% (8/35) of LC neurons, respectively. Pentobarbital (100 μM) inhibited 50% (5/10) of LC neurons. All neurons activated by NMDA ($n = 8$) and glutamate ($n = 3$) were suppressed by ketamine (40 μM), but fentanyl (1 μM) only suppressed 60% (3/5) of spontaneously active and 75% (3/4) of glutamate-activated neurons. Identical LC neurons were inhibited by various combinations of noradrenaline, 5-HT, pentobarbital, ketamine, and fentanyl. The results suggest that clinically relevant concentrations of anesthetics and opioids modulate the activity of LC neurons induced by biogenic amines, excitatory amino acids, and acetylcholine.

Key words: Rat, Nucleus locus coeruleus, Pentobarbital, Ketamine, Fentanyl, Noradrenaline, Serotonin, *N*-methyl-D-aspartate, Glutamate, Carbachol

Introduction

Although all anesthetic drugs are classified as central nervous system (CNS) depressants, the mode of action is divergent among drugs and components of excitation and depression are variously involved [1]. Diversity of action is also observed with other drugs such as opioids. Three major factors appear to affect the mode of action of the drugs: (1) the site of CNS where the agents act upon various types of receptors and ion channels, (2) the concentration of drugs which produce pharmacological effects on the target area, and (3) the background excitability which is modulated by various inputs to the target area with different neurotransmitters.

To study the direct effects of anesthetics and opioids on the CNS, experiments should be performed under the control of the above mentioned factors. We adopted the nucleus locus coeruleus (LC) of the rat as a target area and used an *in vitro* slice preparation for the following reasons. First, the nucleus LC is a restricted area, comprised of only 1400–1600 cells in rats [2,3]. Most of the LC neurons contain noradrenaline (NA) as a neurotransmitter and dopamine-β-hydroxylase as a synthesizing enzyme. Thus, the nucleus LC is relatively homogeneously organized. Second, an *in vitro* preparation allows complete elimination of systemic effects of the drug and permits a study of the direct effects of putative transmitters and drugs on central neuronal activities when they are added to the bathing medium. Third, the nucleus LC receives multi-modal innervation from neurons containing excitatory amino acids (EAAs), NA, 5-hydroxytryptamine (5-HT) and acetylcholine [4]. Activation of these neurons greatly influences the background excitability of the nucleus LC.

In the present study, we firstly described the basal firing patterns of LC neurons induced with or without EAAs and a cholinomimetic drug, carbachol. Secondly, we investigated the effect of biogenic amines such as NA and 5-HT on the activity of LC neurons. Thirdly, we analyzed the effect of anesthetics (pentobarbital and ketamine) and an opioid (fentanyl) at clinically relevant concentrations. We elucidated that the effects of anesthetics and fentanyl were variable in the nucleus LC.

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Materials and methods

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Young Wistar rats (10 days old; body weight, 20–30 g Institute for Animal Experimentation, Hokkaido University School of Medicine) were anesthetized with 3% halothane in oxygen. The animals were then decapitated, and the brains were rapidly removed and placed in the ice-old oxygenated physiological medium. The medium adjusted to pH 7.4, had the following composition (mM): NaCl, 145; KCl, 4; NaH_2PO_4, 1.25; $MgCl_2$, 1.5; $CaCl_2$, 2; glucose, 10; and HEPES, 6. The brains were trimmed, and 400 μm coronal brainstem slices containing the locus coeruleus were cut with a vibrating slicer (DTK-1000, Dosaka EM, Tokyo, Japan). Two or three successive slices were soaked in the same medium at 4°C until use. Under this condition, slices were kept viable for more than 10 h. The slice was transferred to the recording chamber which was perfused with oxygenated medium at a constant rate of 3 ml/min. The chamber was gradually warmed up from 4°C to 30°C by a thermoelectric heating device. All recordings reported here were obtained at 30°C. The LCs were easily identified with transmitted light as relatively translucent oval areas in the upper pons on the lateral borders of the central gray and the fourth ventricle at the level just anterior to the genu of the facial nerve.

Standard extracellular single unit recording techniques were used in these experiments. A glass microelectrode filled with 3M NaCl (resistance, 10–20 MΩ) were positioned under visual control using a stereomicroscope. Electrical signals were led to a high-input impedance amplifier (Duo-773, WPI, Sarasota, Fla.), and were displayed on an oscilloscope (2210, Sony-Tektronix, Tokyo, Japan). Unitary discharges were counted over 10-s periods with the aid of a window discriminator (121, WPI). One slice was used for no more than 3 h to obtain good recording.

Drugs were applied by a fast bath-application system named the "Y-tube" system [5,6]. The test drug was applied from 10 s to 5 min while unitary LC activity was continuously recorded. While drugs were not applied, the physiological medium was continuously perfused through the Y-tube system to the chamber. Drugs tested were *N*-methyl-D-aspartate (NMDA, Sigma Chemical, St. Louis, Mo.); sodium -L-glutamate-mono, carbachol, noradrenaline, serotonin-creatinine sulfate (Wako Pure Chemical, Tokyo, Japan); ketamine hydrochloride, fentanyl (Sankyo Chemical, Tokyo, Japan); and sodium pentobarbital (Nacalai Chemical, Tokyo, Japan). NMDA was first applied to identify LC neurons because most of the LC neurons were silent in this slice preparation. For analyses, we selected the neurons which discharged continuously by application of 250 μM glutamate and/or 1–2 mM carbachol.

Results

Excitatory effects of EAAs and a cholinomimetic agent on LC neurons

Recordings were obtained from 38 LC neurons. Of these, 14 neurons discharged spontaneously. LC neurons exhibited a triphasic waveform consisting of the initial positive component followed by a negative component and a second positive deflection. The firing frequency of spontaneous discharges ranged from 0.2 to 2 spikes/s. Once spontaneous firing was suppressed by application of the test drugs, a washing time of more than 10 min was usually required for recovery. A drug effect was considered significant if the firing frequency of the recorded cell decreased more than 20% of the basal control value.

Bath application of NMDA (50 μM) induced regular repetitive firing in all neurons (Figs. 1 and 2), the firing frequency ranged from 1 to 10 spikes/s (5 ± 2 spikes/s, mean ± SD). The latency of NMDA-induced excitation varied from 1 to 10 s depending on the neuron. Effects of NMDA were reproducible; successive applications of the drug (up to 10 times) elicited the same discharge pattern. This permitted us to confirm that the electrode was still holding the neuron when the drug-induced suppression of the neuronal activity was long-lasting.

Glutamate (250 μM) produced firing in 12 out of 19 silent neurons that had been activated by NMDA, although the firing pattern was less regular than that induced by NMDA (Figs. 1–3).

A cholinomimetic drug, carbachol at a concentration of 1 to 2 mM, elicited discharges in 7 out of 12 silent neurons that had been activated by NMDA (Fig. 1). As carbachol-induced firing was irregular, only a few LC neurons could be analyzed (Figs. 2 and 4).

Effect of NA and 5-HT on the activity of LC neurons

NA at a concentration of 50 μM suppressed the activity of LC neurons in 15 out of 32 neurons that had been successfully activated by NMDA (Figs. 2–4). No effect was seen in 12 LC neurons. Three of 8 spontaneously firing neurons were suppressed by NA, except one which changed the firing pattern from tonic regular discharge to bursting discharges. Four of 13 NMDA-activated cells, 5 of 7 glutamate-activated cells and 3 of 4 carbachol-activated cells were inhibited by NA. Mixed augmented and/or suppressed firing pattern during application of NA was induced in 3 cells activated by either NMDA, glutamate, or carbachol (Fig. 4).

5-HT at concentrations of 1–5 μM suppressed 8 of 35 neurons that had been activated by NMDA (Figs. 3 and 4): 1 of 9 spontaneously active, 1 of 16 NMDA-activated, 4 of 7 glutamate-activated, and 2 of 3 carbachol-activated LC neurons. In two spontaneously

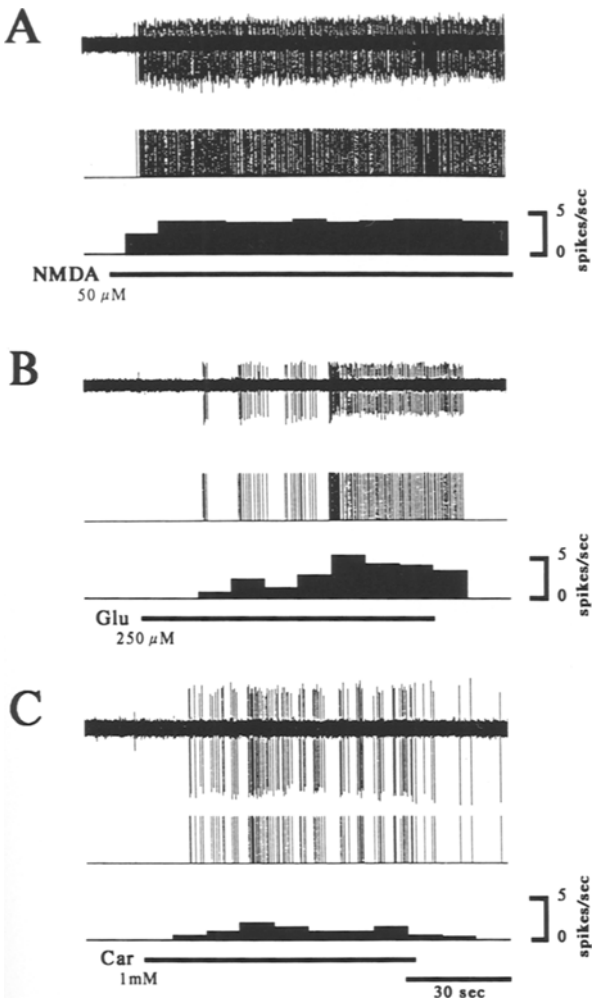

Fig. 1A–C. Activation of the silent locus coeruleus (LC) neurons by excitatory amino acids and carbachol. **A** *N*-methyl-D-aspartat (*NMDA*) (50 μM) produced a tonic regular firing. **B** Glutamate (*Glu*, 250 μM) initially evoked bursting discharges which matured to tonic regular firing. **C** Carbachol (*Car*, 1 mM) produced irregular discharges. Records **A**, **B**, and **C** were obtained from different animals. The *upper* traces are row action potentials; the *middle*, spikes obtained from a window discriminator; and the *bottom*, pulse-count histograms (bin width, 10 s). Application of the drugs is indicated by *bars*

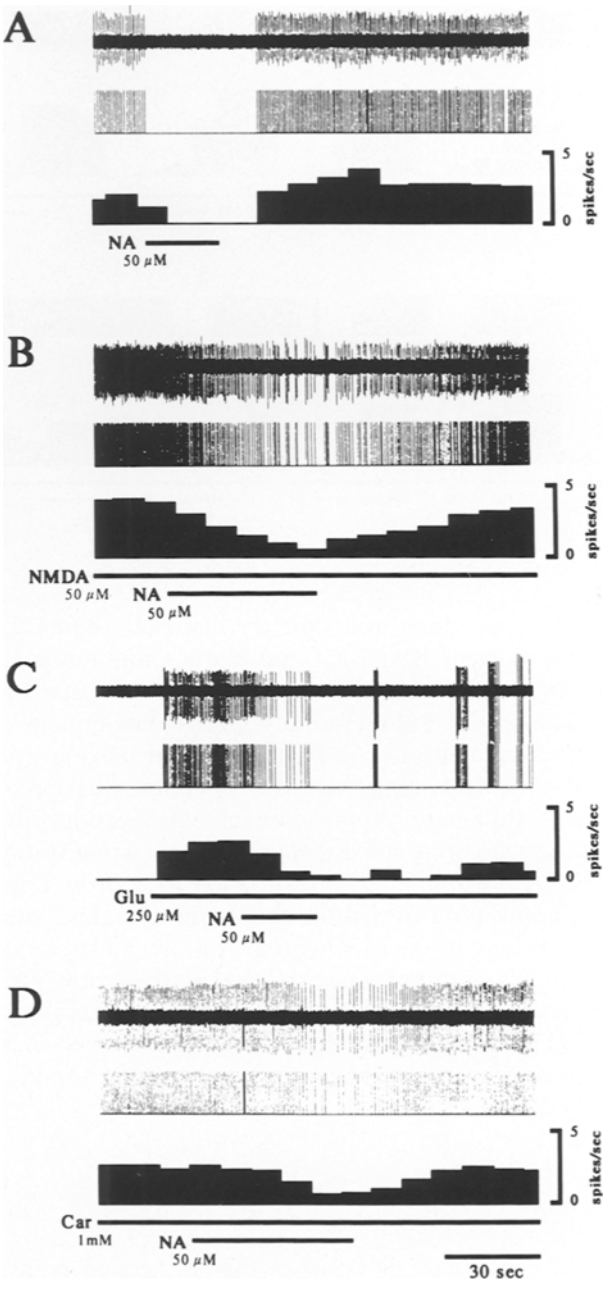

Fig. 2A–D. Inhibitory effects of noradrenaline on the activity of LC neurons. **A** application of noradrenaline (NA, 50 μM) completely suppressed spontaneous firing. **B**, **C**, and **D** Concomitant application of NA decreased the firing frequency in LC neurons activated by NMDA, Glu and Car, respectively

active neurons, 5-HT increased the firing frequency from 2 to 5 spikes/s.

Effects of anesthetics and fentanyl on the activity of LC neurons

Pentobarbital (100 μM) decreased the firing frequency of LC neurons in 5 of 10 neurons that had been activated by NMDA (Figs. 3 and 4): 1 of 2 spontaneously active, 1 of 5 NMDA-activated, 2 of 2 glutamate-activated, and one carbachol-activated LC neuron. Ketamine at a concentration of 40 μM clearly suppressed firing in all LC neurons which were activated by EAAs, NMDA ($n = 8$) and glutamate ($n = 3$; Figs. 3A and 4). One of the 2 neurons activated by carbachol was suppressed by ketamine. Fentanyl suppressed 3 of 5 spontaneously active neurons and 3 of 4 cells activated by glutamate (Figs. 3B and 4). Fentanyl never suppressed NMDA- and carbachol-activated neurons.

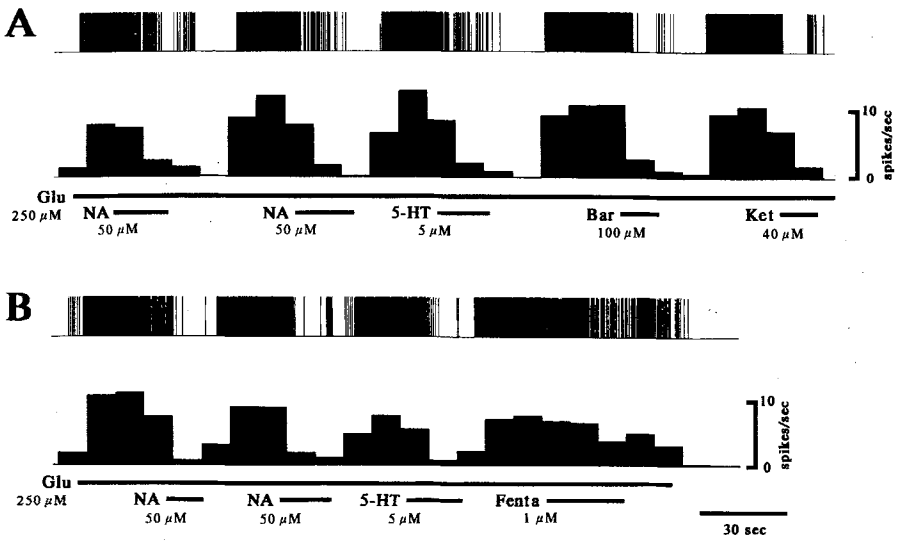

Fig. 3. Effects of noradrenaline, serotonin (5-HT), anesthetics, and fentanyl on glutamate-induced firing. *Top* records are spikes obtained from a window discriminator. *Bottom* records are pulse-count histograms. Application of the drugs is indicated by *bars*. Records **A** and **B** were obtained from different slices of the same animal. Time scale in **B** is also applied to **A**

The decreases in firing frequency by various sequence of applications of NA, 5-HT, anesthetics, and fentanyl were observed in three identical neurons activated by glutamate. Figure 3 shows representative data obtained from the same neurons. An LC neuron shown in Fig. 3A fired at a relatively high frequency by continuous application of glutamate. Application of NA reproducibly suppressed the firing activity of this neuron. Application of 5-HT also exhibited suppression in this neuron. Following application of pentobarbital and ketamine, the firing of this neuron was inhibited. Another LC neuron recorded in a different slice exhibited inhibition by NA, 5-HT, and fentanyl (Fig. 3B). A similar interaction of NA, 5-HT, anesthetics, and fentanyl was observed in 5 NMDA-activated, 2 carbachol-activated, and 1 spontaneously active LC cell.

Discussion

The LC noradrenergic neurons that project to many regions of the CNS [7–9] have been implicated in a wide range of behavioral and physiological processes, including the sleep-wake cycle, cardiovascular regulation, nociception, attention, and learning [4,7,10]. Thus the LC has been hypothesized to play an important role in producing an anesthetic state [11,12].

In anesthetized animals, LC neurons discharged spontaneously in a slow, regular fashion [7,13]. It was reported that LC cells spontaneously fired at a frequency of 0.2–5 Hz in slice preparations obtained from adult rats [14]. In our study, 37% of LC neurons were spontaneously active at a frequency of 0.2–2 Hz. The relatively low firing frequency, however, may be due to our experimental conditions. We used brains from 10-day-old rats and kept brainstem slices at a relatively low

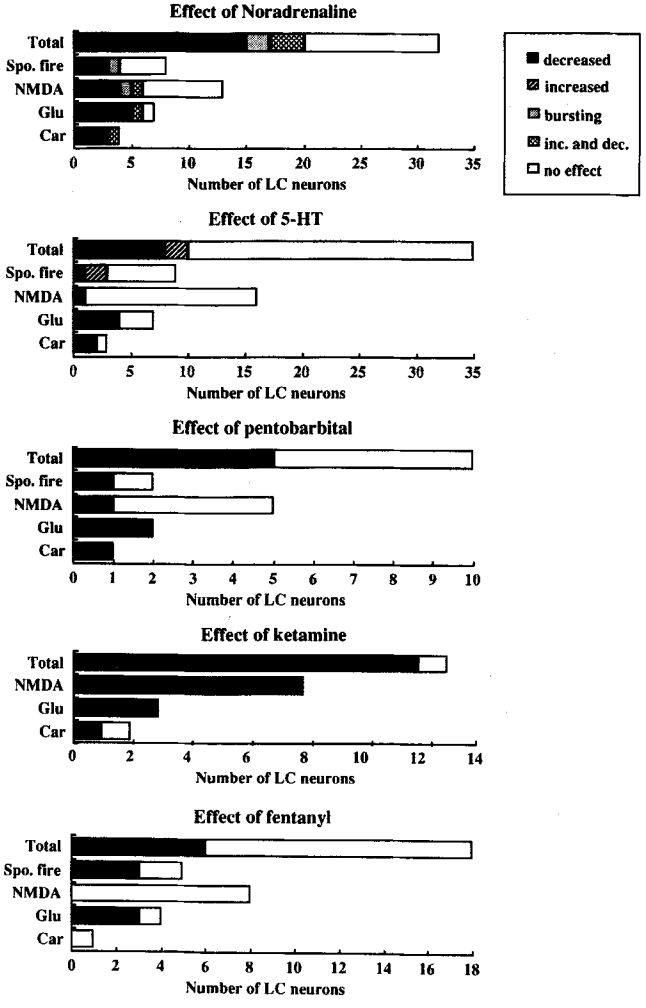

Fig. 4. Summary of the effects of noradrenaline, 5-HT, pentobarbital, ketamine, and fentanyl. Each *bar* indicates the number of neurons which showed various firing characters by application of test drugs. Changes in firing character are indicated by different characters in the box. On the *left*, how LC neurons were activated is indicated. *Top bar* shows the overall effect of each test drug

temperature (30°C) to prolong the viability of the preparation. However, bath application of NMDA produced tonic, regular, and reproducible firing activities in all neurons in the present experiment. Kimura and Nakamura [15] reported that the majority of LC neurons in the urethane-anesthetized neonatal rat were not spontaneously active; however, they were excited by nonnoxious as well as noxious sensory stimuli. Although glutamate also produced activity in 63% of the LC neurons tested, the firing was less regular than that induced by NMDA. This may be attributed to the combined activation of NMDA and non-NMDA receptors of the LC neurons by glutamate. Olpe et al. [16] reported that LC cell activation was greater with NMDA than with glutamate.

As to the acetylcholine receptor, 58% of the LC neurons tested showed firing activity following application of carbachol. These results support immunocytochemical, autoradiographic, and physiological studies [4,7,10,17–21] which revealed that most of the LC neurons expressed EAA receptors, especially NMDA type, and acetylcholine receptors. It has been reported that acetylcholine acts on m_2-muscarinic receptors to excite rat LC neurons [22].

Pentobarbital clearly suppressed firing in LC neurons activated by glutamate, although only a few were analyzed. Barbiturate anesthesia might be due in part to a change in activity in the LC or to a change in noradrenergic transmitter release from this pathway to the cortex [23]. Ketamine, a noncompetitive NMDA receptor antagonists [6,24,25], suppressed the firing of LC neurons induced by glutamate and NMDA at a clinically relevant dose of 40 μM.

The present paper is the first report showing the suppressive effect of fentanyl (1 μM) on spontaneous LC activity and on that induced by glutamate in the slice preparation. The plasma concentration of fentanyl in high-dose fentanyl anesthesia is 6–10 μM [26]. It is reported that fentanyl decreased catecholamine metabolism measured by *in vivo* voltametry in the rat LC [27]. Local application of morphine to LC neurons *in vivo* potently decreased their spontaneous discharges [28], as did intraventricularly administered morphine [29]. Studies *in vitro* have shown that opiates act at μ receptors to directly inactivate LC neurons by hyperpolarization [30,31]. It has been proposed that opiate and alpha-2 agonists depress the firing of LC neurons by inhibiting adenylate cyclase via a guanine nucleotide regulatory protein [32].

NA and 5-HT have received particular attention as potential CNS modulators and a wide variety of actions has been reported for them. NA, a major neurotransmitter of LC neurons, showed suppression in about 50% of neurons. In some neurons activated by NMDA, glutamate, and carbachol, NA both increased and decreased the firing frequency. Reports show that activation of alpha-2 receptor causes a hyperpolarization of LC neurons by increasing membrane potassium conductance [30,32,33]. It is most probable that NA suppressed LC activity by activating alpha-2 receptor of the neurons.

Very few LC neurons, either spontaneously active or activated by NMDA, were suppressed by 5-HT, while 57% of LC neurons activated by glutamate were inhibited by 5-HT. 5-HT suppression was observed in 2 of 3 carbachol-activated LC neurons. The present results were partly consistent with those reported by Aston-Jones et al. [4]. They reported that 5-HT reliably attenuated responses of LC neurons to glutamate and kainate but not of acetylcholine. 5-HT had no consistent effect in spontaneous LC discharges. Since there still exists local synaptic interactions inside and outside the nucleus LC even in the *in vitro* slice preparation, a modulatory action of NA and 5-HT on the activity of LC neurons might potentiate the inhibitory effects of anesthetics and opioids.

In summary, LC neurons, activated by EAAs, were influenced by the biogenic amines NA and 5-HT. Some LC neurons under the control of EAAs and biogenic amines were inhibited by clinically relevant concentrations of anesthetics and fentanyl. These results suggest that the depressant effects of anesthetics and opioids might be reduced by the inputs to LC neurons utilizing EAAs and acetylcholine as neurotransmitters, and be facilitated by those utilizing biogenic amines. It is also conceivable that the central action of anesthetics and opioids is modified with various drugs that influence the concentration of intrinsic neurotransmitters in the CNS.

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