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Original Article

The effects and mechanism of substance P in sedative and hypnotic of isoflurane mice

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Abstract: Objective: To investigate the changes of serum substance P (Substance P, SP) content and its effects on sedation and hypnosis after mice inhale Isoflurane. Methods: 45 mice were randomly divided into Isoflurane (Isoflurane, Iso) group, Dexmedetomidine (Dexmedetomidine, Dex) group and Isoflurane combined with substance P receptor antagonist agent (Antagonist of Substance P, Iso+A-SP) group; using ELISA method to detect serum substance P content of groups of mice, applying analepsia experiment to record sleep time of groups of mice, applying Hot-plate and writhing test to record threshold of pain in Hot-plate method and writhing frequency of groups of mice. Brain stereotaxic, nucleus spile, micro-injection and polysomnography were used to apply in the blank control group (PBS), substance P group (SP group), substance P receptor antagonist agent group (A-SP group), 3-Mercaptopropionic acid group (3-MP group) and substance P+3-Mercaptopropionic acid group (SP+3-MP group) for observing the mechanism of action of SP on sleep of mice. Results: Iso group had the strongest sedation and the Iso+A-SP group was the weakest. For writhing frequency, Dex group and Iso+A-SP group were significantly more than that of the Iso group, while Iso+A-SP group was more than that of the Dex group; after medication, the threshold of pain in Iso+A-SP group had no significant difference compared with medication before, while Iso group and Dex group were prolonged pain threshold; Iso group and Dex group pain threshold were the longest when the medication for 10 minutes, the above difference was statistically significant ($P<0.05$); the sleep time of Iso group was longer than that of Iso+A-SP group and Dex group, the sleep time of Iso+A-SP group was the shortest, and there was statistical difference ($P<0.05$); before medication, there was no significant difference in the concentration of substance P in each group; after medication, Iso group and Iso+A-SP group of P substance concentration difference was quite little, but Iso group and Iso+A-SP group were significantly higher than the Dex group, the difference was statistically significant ($P<0.05$). Compared with the control group (PBS group), sleep time of micro-injection SP (SP group) in the ventrolateral preoptic area (vLPO) in mice was significantly increased while the awakening time was significantly decreased; compared with the control group (PBS group), sleep time of micro-injection A-SP (A-SP group) in vLPO was significantly decreased while the awakening time was significantly increased; compared with the 3-MP group, in the vLPO micro-injection of gamma aminobutyric acid (GABA) compounded key enzyme inhibitors substance P+3-Mercaptopropionic acid group (SP+3-MP group), the sleep time was significantly decreased while the awakening time was significantly increased, the differences mentioned above were statistical significance ($P<0.05$). Conclusions: Sedative and hypnotic effects of Isoflurane had close relationship with the level of substance P in the body, these effects might be achieved by the substance P, which mediated GABA neurons in vLPO.

Keywords: Substance P, isoflurane, sedative, hypnotic

Introduction

At present, general anesthesia is the most commonly used method of anesthesia in clinic, in which sedative and hypnotic is an indispensable part. Endogenous neuropeptide substance P (Substance P, SP) is a kind of nerve signals and information transmission of the substance, as one of the earliest discovered tachykinins, it plays a very significant role in the nervous system [1]. SP signaling is a kind of harmulness information, which is involved in the occurrence of pain. At the same time, the SP also interacts with other substances in the nervous system and neurotransmitters, and its mechanism is so complicated that so far its clear results has not been achieved [2]. In recent years, some studies have shown that after peripheral injec-

tions SP can affect the sleep quality of healthy people [3], which has shown that the SP has been also involved in the regulation and maintenance of sleep while anesthesia and may have the approximate location and mechanism of sleep. Isoflurane is a clinical drug which is widely used in the general anesthesia and induction [4], at present in the process of anesthesia the effects of Isofluraneon serum SP in related literature reports have no precise definition, but in the process of clinical application, taking isoflurane as surgical anesthesia induction and maintenance of clinical information of drugs, the incidence of infection and traumatic surgery complications is significantly lower than that in other general anesthesia [6]. Therefore, some scholars infer that Isoflurane affect the levels of SP in human body, it may also be one of Isoflurane sedative-hypnotic mechanisms [7]. This article applied Isoflurane to anesthesia induction and maintenance, studied the mechanism of SP in sedative-hypnotic, provided the experimental basis in order to further clarify the mechanism of Isoflurane in sedative-hypnotic, and provided experimental basis for clinical medication.

Data and methods

Reagent and instrument

Isoflurane (25626Z8) was produced by British abbott pharmaceutical Co.LTD., Dexmedetomidine (H20110097) was applied by Sichuan Guorui pharmaceutical Co.LTD. SP receptor antagonist Spantide (0.00125%) (Sigma company); gamma-aminobutyric acid (GABA) Synthetase inhibitor 3-mercaptopropionic acid (3-MP) (concentration: tendency for 0.5 L0.5 mmol/L) (Sigma Company); SP (concentration: 10 nmol/L) was bought from Sigma company; YLS-6B Intelligent Hotplate (Huaibei Zhenghua biological equipment Co.LTD.); Mouse Substance P, SP ELISA Kit (ADI Company, USA), etc.

Experimental animals

45 mice of Kunming species weight 18-25 g, average (20.36±2.31) g, all of them were provided by our animal experiment center. Animal production licensee was SCXK (Shanghai) 2012-0002, and animals use licensee was SYXK (Shanghai) 2012-0002. Two days before the beginning of the experiment, the mice were placed to experimental environment for breeding, eating food and drinking water freely, in order to make them be familiar with breeding environment.

Experiment methods

According to random number table method, mice were divided into 3 groups randomly, each group was 15 mice, namely the Isoflurane (Isoflurane, Iso) group, the Dexmedetomidine (Dexmedetomidine, Dex) group and the Isoflurane combination SP receptor Antagonist (Antagonist Substance P, A-SP) groups respectively. The mice of Iso group and Iso+A-SP group were put into the closed Hot plate to do anesthesia induction, and air outlet end was connected multifunction gas monitoring instruments while air inlet end was connected with the an aesthetic machine. The mice were kept in spontaneous respiration, the Isoflurane vaporizers were regulated to the concentration, the oxygen flow rate in container were maintained at 500 ml/min and the temperature was kept at 37°C. The mice of Dex group were performed intraperitoneal injection with the Dexmedetomidine 50 μg/kg.

Substance P effects on isoflurane mice's hypnotic effect

Hypnosis models of groups were established with Isoflurane or Dexmedetomidine, 1 minute after the disappearance of Righting reflex, and then substance P antagonists was injected intravenously to Iso+A-SP group's mice. Time from righting reflex to recovery of righting reflex of each group of mice was recorded, namely sleep time.

Hot plate and writhing test pain threshold

Pain thresholds of each group were measured twice for mice before the establishment of Hypnosis model (The temperature of the hot plate was adjusted to 55°C, and the mice were placed on an intelligent hot plate instrument. The time of the mice from the foot touching the hot plate to start to lick the hind foot was recorded, as the pain threshold in mice. Pain thresholds of mice less than 5 s and higher than 30 s were to be excluded, the observation period was set to 1 minute in order to prevent mice's foot scald) and the average of the two result was regarded as the pain threshold. Each group after administration of hypnotic 5 min,

Table 1. Comparison of sedation degree in the three groups of mice

Observation indcator		Iso group	Dex group	Iso+A-SP group
Writhing times (n)		13.69±2.36	26.36±3.69*	44.23±4.39*,#
Pain threshold before medication (s)		9.32±0.97	9.87±1.07	9.63±1.02
The pain threshold of different time of mice after medication	5 min	17.36±2.36	14.02±2.36*	8.86±0.69*,#
	10 min	34.65±2.36	21.36±2.36*	8.69±0.97*,#
	20 min	22.91±6.23	15.36±2.31*	9.02±1.23*,#
	30 min	21.36±2.21	9.85±1.21*	9.36±1.36*

Note: Compared with Iso group, *P<0.05; and compared with Dex group, #P<0.05, and differences have statistical significance.

Table 2. Comparison of sleep time in the three groups of mice

Group	Sleep time (min)
Iso group	28.34±6.21*
Dex group	12.36±2.31#
Iso+A-SP group	0.15±0.03

Note: Compared with Iso group and Iso+A-SP group, *P<0.05; and compared with Iso+A-SP group, #P<0.05, and differences have statistical significance.

Table 3. Comparison of substance P levels in three group of mice

Group	Substance P levels in blood	
	Before medication (pg/ml)	After medication (pg/ml)
Iso group	288.36±26.35	890.36±26.32*
Dex group	291.32±28.21	326.31±25.21
Iso+A-SP Group	287.32±28.14	939.36±25.49*

Note: compared with Dex group, *P<0.05.

10 min, 20 min, 30 min, was used to measure the pain threshold of mice, and then the mice were performed intraperitoneal injection of 1.0% acetic acid 10 ml/kg to cause pain. 15 minutes after the mice were injected, the frequency of writhing was recorded.

Concentration of the substance P detected

1 hour after each group's establishment of hypnosis model respectively, blood was drawn from the tail vein of mice according to the kit instructions of SP by enzyme-linked immunosorbent assay measured.

Substance P influence on the sleep of mice

50 mice were selected and randomly divided into control group (PBS solution, Control group), SP Group (concentration: 10 nmol/L, SP Group), SP receptor antagonist group (concentration: 10 nmol/L, A-SP group), 3-mercaptopropionic acid group (3-MP group) and SP+3-mercaptopropionic acid group (SP+3-MP group), 10 mice in each group. Heads of the mice were fixed to the SN-2 type of stereo-positioner of brain, their skulls were exposed. The mice cortex brain electrical activity was recorded. The silver electrode was inserted into the bilateral neck muscle to record EMG activity. Brain stereotaxic atlas was used to insert a diameter of 23 gauge (0.6 mm, OD) stainless steel guide tube into the bilateral ventrolateral preoptic area (VLPO). The guide tube was from the top vLPO 1 mm, for microinjection of drugs into the vLPO use. Cannula and recording electrodes were fixed with dental cement on the skulls. When the awake mice were under the condition of being recorded, 0.1 ul drug was injected into each intubation of the nuclear group, the mice were acted hypnosis with Isoflurane and sleep cycle of each group of mice was continuously recorded and analyzed, as well as the polysomnography was used to record awakening period (W) Light slow wave sleep (SWS1), deep slow wave sleep (SWS2), slow wave sleep (SWS) and paradoxical sleep phase (PS), wherein sum of SWS1, SWS2 and PS were total sleep time (TST).

Statistical methods

Use SPSS17.0 software for statistical analysis of data of the results of this study, measurement data was expressed as mean ± standard deviation of the way, and groups were compared by using t test. When P<0.05, the difference was statistically significant.

Results

Comparison of level of sedation

From the data in **Table 1**, Iso group sedation was the strongest, followed by Dex group, Iso+

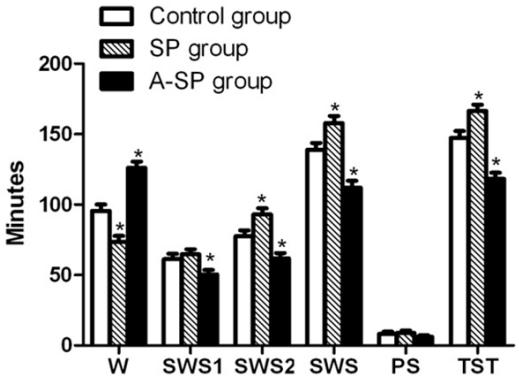

Figure 1. Effects of micro injection substance P and substance Preceptor antagonist into vLPO on the sleep of mice (min, Mean ± SD). *Compared with control group, P<0.05.

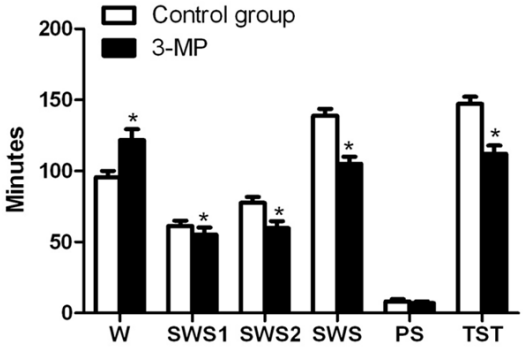

Figure 2. Effects of micro injection 3-MP into vLPO on the sleep of mice on the second day (min, Mean ± SD). *Compared with control group, *P<0.05.

sedation A-SP group was the weakest. The writhing frequecy of Dex group and Iso+A-SP group were more than that of Iso group, while writhing frequecy of Iso+A-SP group was more than that of Dex group; after medication, pain threshold of Iso+A-SP group had no significant differences compared with no medication, but pain threshold of Iso group and Dex group were prolonged; and pain threshold of Iso group and Dex group were the longest after 10-minute medication. Above differences had statistical significance, P<0.05.

Comparison of sleep time

From the data in **Table 2**, sleep time of Iso group was longer than that of Dex group and Iso+A-SP group, while sleep time of Dex group was longer than that of Iso+A-SP group. The difference was statistically significant, P<0.05.

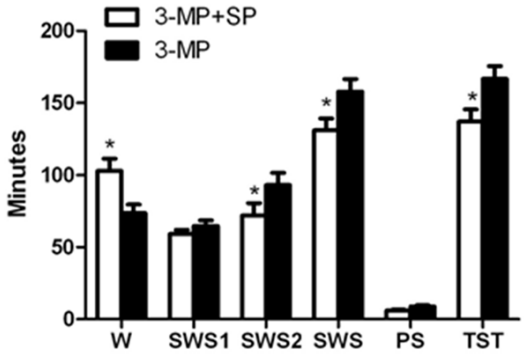

Figure 3. vLPO microinjection of SP+3-MP on the role of sleep in mice (min, Mean ± SD). *Compared with the 3-MP group, *P<0.05.

Comparison of substance P levels

From the data in **Table 3**, before medication, the difference of substance P levels in each group had no statistical significance (P>0.05). After medication, Iso group and Iso+A-SP group substance P levels varied little, which were both significantly higher than that of DEX group, and the difference had statistical significance (P>0.05).

Effects of micro injection SP and A-SP into vLPO on the sleep of mice

Compared with control group, after micro injection SP into the bilateral vLPO, wakefulness cycle of mice decreased by 23% while SWS1, SWS2, SWS, PS and TST increased respectively by 5.5%, 19.8%, 12.6%, 7.2% and 13.1%. Compared with control group, after micro injection A-SP into the bilateral vLPO, wakefulness cycle of mice decreased by 32% while SWS1, SWS2, SWS, PS and TST respectively decreased by 17.7%, 20.5%, 19.3%, 26% and 19.7%, and the difference were statistically significant (P<0.05), see **Figure 1**.

Effects of micro injection 3-mercaptopropionic acid (3-MP) (gamma aminobutyric acid (GABA) synthetase inhibitor) into vLPO on the sleep of mice

Compared with control group, after micro injection 3-MP into the bilateral vLPO, sleep time of mice decreased slightly on the first day, but it significantly decreased on the second day (113±14.2 min vs 133.7±14.8 min), and wake-

fulness increased significantly (127.5±16.3 min vs 96.3±15.7 min). The differences had statistical significance (P<0.05). Sleep and wakefulness gradually restored to normal level on the third or fourth day. Compared with the control group, on the second day, the wakefulness cycle of micro injection 3-MP increased to 27.5%, SWS1, SWS2, SWS, PS and TST decreased respectively to 10%, 22.9%, 15.5%, 14% and 15.6% (see **Figure 2**).

The role of vLPO microinjection of SP and 3-MP on mouse sleep

VLPO microinjection of 3-MP was taken as a control, 3-MP and SP after microinjection of vLP0, it was found that wakefulness of mice increased to 39.9% while SWS1, SWS2, SWS, PS and TST respective decreased to 8.8%, 22.8%, 17%, 31.6% and 17.8%, see **Figure 3**.

Discussion

Patients' undergoing loss of consciousness caused by application of general anesthesia (sedative, hypnotic) has been a history of more than 160 years, but the generation of awareness, maintenance of wakefulness and mechanism of loss of consciousness was still poorly understood. Now that there are different roles of anesthetics such as sleep, loss of consciousness, muscle relaxation, memory impairment, etc., there may be relatively specific sites and pathways being involved in [8]. As Isoflurane anesthesia is a kind of commonly used clinical drugs, many scholars studied the action mechanism of Isoflurane in-depth. Studies have shown that Isoflurane could suppress the uptake route of synaptic of serotonin and decreased the combined amount of acetylcholine in the brain [9]. The inhalation of Isoflurane was inhaled, which could also suppress the increasing of the calcium in neurons [10]. Isoflurane inhibition function of nicotinic receptor was mainly achieved through the regulation of calcium ions, but the regulation of nicotinic receptors was not only affected by calcium-ions, but also had a certain relevance with the role of acetylcholine and dopamine and other neurotransmitters in the body, and Isoflurane had a facilitating role on secretions of these neurotransmitters [11]. In addition, SP was released to increase the role of postsynaptic neurons, change the electrical activity of neurons, and thus play a role in the transmission process of pain [12, 13].

From the results of this study, before treatment, concentration of SP in mice blood of three groups had no difference; after the treatment, SP concentration significantly increased in blood of Iso group and Iso+ group, and in these two groups, SP concentration changed little. It proved that SP did play an important role in pain transmission process, A-SP did prevent SP binding to its receptor and resulting in little change in the level of SP. From the sleep time, mice of Iso group had the longest sleep time, mice in Iso+A-SP group had the shortest sleep time, mice of Dex group was in the middle place, indicating that compared with Dex group Isoflurane contributed more to sleep of mice, the hypnotic effect also had a close relationship with the SP level. Besides, Iso+A-SP group had the shortest sleep, indicating that the use of A-SP could be suppressed by its SP-SP binding, so hypnotic effect could not work, thus further affecting sleep time of mice [14-16]. From the writhing frequency of mice, this theory was also supported.

From the results of this study, at different times after treatment, the pain threshold of mice were different. Iso group and Dex group were in the treatment to achieve the best sedative effect 10 minutes later, and Isoflurane in the medication also still had a calming effect 30 minutes later, while Dexmedetomidine was substantially no sedative effect 30 minutes after, so sedative effects for Isoflurane of mice were better. Some studies [17-19] used Isoflurane to do general anesthetic, and then analyzed the changes of TNF-α, IL-6 and SP content in patients' serum and the possible factors. It was concluded that Isoflurane had a significant effect on the TNF-α, IL-6 and SP content in serum, and the content of SP also increased significantly during the whole testing process [20].

The occurrence of sleep involved a complex mechanism of the interaction among various factors and several brain centers. Studies have been reported [21] that the expression of fos protein in vLPO neurons of rat hypothalamus increased in sleep state and decreased in wake state. The vLPO neurons' discharge frequency increased in sleep state and was proportional to the depth of sleep. Sleep time decreased significantly when vLPO neurons were injured, and these studies indicated that vLPO was an important part of the occurrence of human

sleep. 80% of neurons contained inhibitory GABA, and they sent out nerve fibers to the waking center such as the brainstem raphe nuclei, tuberomammillary nucleus, and locus coeruleus that were behind the hypothalamus, which caused the inhibition of sleep. SP is a neuropeptide substance found in the body. It is one of the tachykinin family members, and the SP neurons and its receptors distribute widely in peripheral and central nervous systems. Previous reports indicated that SP played a vital role in gastrointestinal motility, cardiovascular events and pathogenesis of pain. Recently, some studies have shown that SP may be involved in sleep regulation processes of the body [23]. The cell bodies of cholinergic neurons in both dorsal pons and midbrain contained substance P and the axonal fibers project to the pontine reticular formation that induces paradoxical sleep. It showed that SP was related to the regulation of body's sleep but there was no direct evidence being provided in this study [24]. In addition, large amounts of SP-positive nerve fiber projections and expression of SP receptors were contained in vLPO. To clarify the role of SPon vLPO sleep regulation in this study, we gave a microinjection of SP into the vLPO, and the results showed that sleep time of the mice was significantly increased while the microinjection of A-SP later had the opposite effect that sleep time was reduced. It showed that SP was essential for vLPO in hypnotic. It was reported [25] that GABAergic neurons of vLPO contained SP receptors, and it speculated that SP might release GABA by binding to the SP receptors of GABAergic neurons in order to increase the sleep time. In order to confirm this point of view, this study chose the 3-MP, a kind of drug which could inhibit a key enzyme in the synthesis of GANG with micro-injection into vLPO. The results showed that sleep time of mice was significantly reduced in the first two days, then the sleep time of mice gradually returned to normal three or four days later. In this study, after the microinjection of 3-MP, SP was injected on the next day, and it showed that the original promotion of hypnotic effect of SP no longer occurred. This indicated that SP achieved the effect of promoting sleep by the GABAergic neurons in vLPO.

To sum up, the sedative and hypnotic effects of Isoflurane is better, and its effects are closely related to the substance P levels in the body. The effects probably are achieved by means of substance P which mediated the GABAergic neurons in vLPO. But it is necessary to be further confirmed by deep research.

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Disclosure of conflict of interest

None.

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