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Research article

Intrathecal L-arginine reduces the antinociception of sevoflurane in formalin-induced pain in rats

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HIGHLIGHTS

- L-arginine reduced the antinociceptive effects of emulsified sevoflurane.
- Emulsified sevoflurane reduced the Fos protein expression in the spinal cord.
- L-arginine attenuated the suppression of sevoflurane in Fos protein expression.

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ABSTRACT

The purpose of this study was to investigate the contribution of spinal nitric oxide (NO) to the antinociceptive effects of emulsified sevoflurane in rats. Formalin tests were used to assess the nociceptive response. Immunohistochemistry was performed to determine the effects of emulsified sevoflurane on formalin-induced changes of Fos-like immunoreactive (Fos-LI)-positive neurons in the spinal cord. We found that emulsified sevoflurane administered intraperitoneally at a dosage of 2.5 ml/kg did not impair motor performance in rats, but it significantly decreased the formalin-induced paw licking time. Furthermore, Fos-LI-positive neurons were mainly found in the ipsilateral dorsal horn after the injection of formalin. The administration of emulsified sevoflurane significantly decreased Fos-LI-labeled neurons. Finally, intrathecal L-arginine alone did not affect the basal pain threshold, but it significantly decreased the antinociceptive response of emulsified sevoflurane against formalin injection and the suppressive effects of sevoflurane on formalin-induced Fos protein expression ($P < 0.05$). These data suggest that spinal cord NO is involved in the antinociception of sevoflurane in rats.

1. Introduction

Sevoflurane is widely used in the clinical setting as an inhalation anesthetic. Many studies have shown that sevoflurane has analgesic properties and that its analgesic properties are mediated by the spinal cord [1,2]. However, the exact mechanisms and pathways of its anesthetic action are not clearly understood.

Previous experiments have shown that noxious stimulation increases the expression of the c-fos proto-oncogene in subpopulations of spinal cord neurons [3,4]. Therefore, the Fos protein, the product of the c-fos immediate early gene, has been used as a marker of activation of nociceptive pathways [5]. There is evidence that the activation of nitric oxide synthase (NOS) with a consequent production of NO plays an important role in the central and peripheral modulation of nociception and the expression of the Fos protein [6,7].

Certainly, NO is involved in the action of volatile anesthesia. However, its role is far from being understood [8–10]. Few studies have been dedicated to understanding the effects of spinal cord NO on the antinociceptive of sevoflurane.

Based on these observations, the goal of this study was to ascertain the role that NO plays at the spinal level in the antinociceptive effects of emulsified sevoflurane using L-arginine.

2. Materials and methods

2.1. Animal preparation

The experiments were performed on healthy Sprague–Dawley rats weighing 250–300 g from the Xuzhou Medical Experiment Animal Center. All experimental protocols were approved by the Ethics

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Committee for the use of experimental animals of Xuzhou Medical University.

2.2. Intrathecal catheter

Intrathecal catheterization was based on the procedure described by Yaksh [11]. Animals were injected intraperitoneally with sodium pentobarbital (40 mg/kg), and every rat received an incision over the cervical spine, and a small puncture was made in the dura mater. PE-10 polyethylene tubing(OD 0.55 mm, ID 0.30 mm) was threaded 7.5 cm into the intrathecal space. This tubing was then sutured in place, and the skin was sutured tight over the tubing. Correct catheter placement was verified by injecting 2% lidocaine 10 μl down the catheter after recovery from general anesthesia. The catheter was considered to be introduced intrathecally if paralysis and dragging of the two legs were observed after the injection. Experiments were started 6 days after the placement of the intrathecal catheter. Rats with neurological disturbances and a negative lidocaine test were excluded from the following experiments [12]. Intrathecal (i.t.) drug administration was accomplished manually using a microinjection syringe (OD 0.5 mm) connected to the intrathecal catheter over a 10 s period in a single injection with a volume of 10 μl followed by a flush of 10 μl normal saline (NS) in awake rats. Intraperitoneal (i.p.) drug administration was performed in a single volume of 0.01 L/kg.

2.3. Formalin test

Formalin-induced pain was produced based on a previously reported method [13]. Briefly, 20 μl of 10% formalin solution was injected subcutaneously (s.c.) under the plantar surface of the left hind paw. The time that animals spent licking the injected paw was monitored during the early phase (phase I) and during the late phase (phase II). Phase I was defined as the period of time from the beginning immediately after formalin injection to the next 5 min. Phase II was defined as the time from the first 5 min immediately after post-formalin injection to the last 50 min. Phase II was proposed to be caused by central sensitization. The phase I response of the formalin test was caused by the direct stimulation of nociceptors by formalin or tissue damage, while phase II was proposed to be caused by subsequent inflammation after formalin injection [8].

2.4. Experimental design

First, we evaluated the effect of L-arginine on antinociception of emulsified sevoflurane on formalin-induced pain. Sixty-four animals were randomly assigned to one of eight groups ($n = 8$ per group). Group NS (rats received i.t. and i.p. injections of NS 10 min before s.c. injection of formalin); group L-arginine 10 μg, 20 μg, and 40 μg (rats received i.t. injection of 10 μg, 20 μg, and 40 μg of L-arginine, respectively, 10 min before s.c. injection of formalin). Group Sev (rats received i.p. injection of 2.5 mL/kg emulsified sevoflurane 10 min before s.c. injection of formalin). Group L-Arg 10 μg + Sev group 20 μg + Sev and group 40 μg + Sev (rats received i.t. injection of 10 μg, 20 μg, and 40 μg of L-arginine 10 min before i.p. injection emulsified sevoflurane and 10 min before s.c. injection of formalin) (Fig. 1).

To further reveal the role of NO in the effects of emulsified sevoflurane, we used the Fos protein as a marker of the activation of nociceptive pathways. A total of 54 rats were randomly divided into 9 groups ($n = 6$): (1) rats in group NS underwent i.t. injection of NS; (2) rats in group Sev underwent i.p. injection of 2.5 ml/kg emulsified sevoflurane; (3) rats in group F underwent s.c. injection of formalin; (4) rats in group NS_{ip} + F underwent i.p. injection of NS 10 min before s.c. injection of formalin; (5) rats in group NS_{it} + F underwent i.t. injection of NS and s.c. injection of formalin; (6) rats in group Sev + F underwent i.p. injection of 2.5 ml/kg emulsified sevoflurane10 min before s.c. injection of formalin; (7) rats in group L-Arg_{it} + F underwent i.t. injection of 20 μg L-Arg 10 min before s.c. injection of formalin; (8) rats in group NS_{it} + Sev + F underwent i.t. injection of NS, i.p. injection of 2.5 ml/kg emulsified sevoflurane 10 min before s.c. injection of formalin; (9) rats in group L-Arg_{it} + Sev_{ip} + F underwent i.t. injection of L-Arg i.p. injection of 2.5 ml/kg emulsified sevoflurane 10 min before s.c. injection of formalin using the fos immunohistochemistry technique, and changes in the Fos expression in the spinal cord were observed (Fig. 2).

2.5. Immunohistochemistry

Based on a previous report [14], one hour after formalin injection, rats were anesthetized with sodium pentobarbital (100 mg/kg. i.p.) and underwent sternotomy. They were then intracardially perfused with 250 ml saline followed by 400 ml 4% ice-cold paraformaldehyde in 0.1 mol/L phosphate buffer (PB). The spinal cord from L_4 to L_5 was removed, post-fixed in 4% paraformaldehyde for 3 h, and subsequently allowed to equilibrate in 30% sucrose in PB overnight at 4 °C. Thirty-micrometer transverse serial sections were cut on a cryostat and stored in phosphate buffered saline (PBS). Tissue sections were performed for Fos protein expression. Tissue sections were washed and incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min, followed by primary rabbit anti-c-Fos antibody (Sigma, 1:600) at 4 °C for 48 h. Then, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at 37 °C for 1 h, then in the avidin–biotin–peroxidase complex (1:100) at 37 °C for 2 h. Finally, the sections were reacted with diaminobenzidine (DAB) for 5–10 min and then rinsed in 0.01 mol/L PBS to stop the reaction, mounted onto gelatin-coated slides, air dried, dehydrated with 70–100% alcohol, cleared with xylene and cover slipped for microscopic observation.

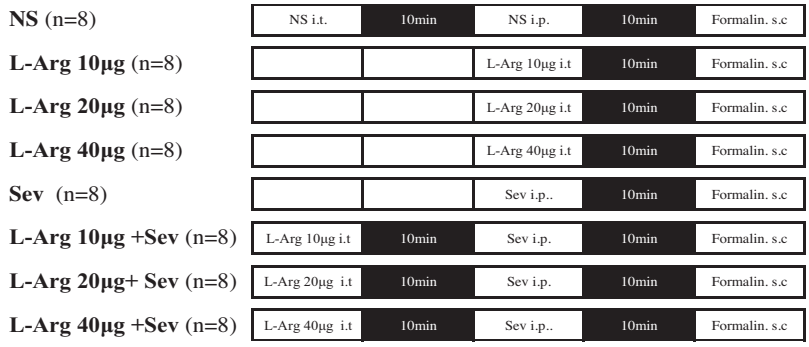

Fig. 1. Paw licking time (PLT).

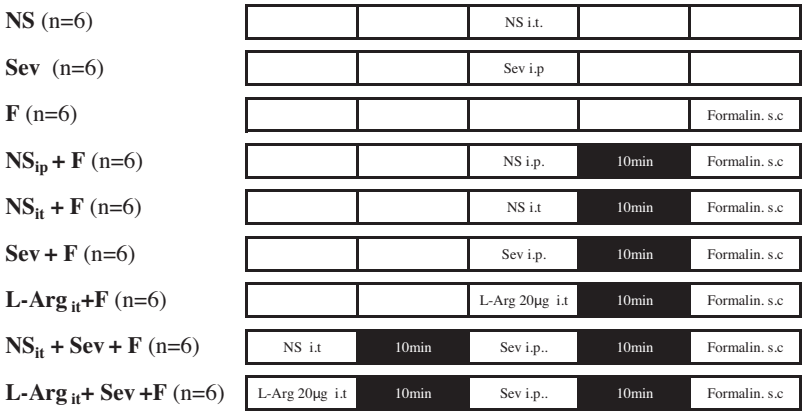

Fig. 2. Fos expression in the spinal cord in rats.

Table 1
Paw licking time (PLT) in each group($n = 8$, $\bar{X} \pm S$).

Group	PLT (s)	
	0 ~ 5 (min)	1 ~ 50 (min)
NS	68 ± 15	192 ± 78
L-Arg 10 μg	76 ± 11	185 ± 24
L-Arg 20 μg	89 ± 18	189 ± 27
L-Arg 40 μg	78 ± 12	178 ± 30
Sev	37 ± 17*	60 ± 12*
L-Arg 10 μg + Sev	40 ± 16*	64 ± 18*
L-Arg 20 μg + Sev	51 ± 12#	129 ± 27#
L-Arg 40 μg + Sev	70 ± 16#	128 ± 29#

* $P < 0.05$ compared with Group NS.
\# $P < 0.05$ compared with Group Sev.

2.6. Counting of positive neurons

To observe the distribution of positive neurons, the spinal cord was divided into four regions: superficial laminae (I–II laminae), nucleus proprious (III–IV laminae), neck of the dorsal horn (V–VI laminae) and ventral laminae (VII–X laminae). We selected 5 sections per rat to count the greatest number of positive neurons in the ipsilateral spinal cord. All positive neurons were counted without considering the intensity of the staining.

2.7. Statistical analysis

The data were presented as the means ± SD and analyzed with SPSS 11.0 statistical software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) and post hoc Tukey's test were used to analyze the differences. *P*-values less than 0.05 were considered statistically significant ($P < 0.05$).

3. Results

3.1. Effects of systemic emulsified sevoflurane (2.5 ml/kg, ip), intrathecal L-arginine or their combination in the formalin test

In NS-treated rats, the duration of paw licking across the early phase was (68 ± 18)s and across the late phase was (192 ± 78)s. The administrations of emulsified sevoflurane 10 min prior to formalin injection significantly reduced formalin-induced licking behaviors during phase I and phase II ($P < 0.05$). L-arginine (10 μg, 20 μg, 40 μg) administered intrathecally alone exhibited no effects on the duration of paw licking, compared with baseline values and control ($P > 0.05$). Pretreatment with 20 μg and 40 μg L-arginine, but not 10 μg, significantly reduced the effects of emulsified sevoflurane on formalin-induced licking behaviors ($P < 0.05$) (Table 1).

3.2. Fos protein expression

There were few Fos-like immunoreactivity (Fos-LI) positive neurons in the spinal cord in the NS group. Systemic emulsified sevoflurane alone had no effects on the expression of Fos-LI-positive neurons in the spinal cord compared with the NS group ($P > 0.05$). Formalin-induced Fos-LI positive neurons were mainly located in the superficial laminae (I–II laminae) and neck (V–VI laminae) of the ipsilateral spinal cord (Fig. 3A). The injection of emulsified sevoflurane 10 min prior to formalin injection could significantly reduce the number of Fos-LI neurons (Fig. 3B), compared with the NS_{it} + F group ($P < 0.05$) (Table 2).

Intrathecal treatment with 20 μg of L-arginine alone had no significant effect on Fos protein expression in the spinal cord but obviously attenuated the suppression of sevoflurane on formalin-induced spinal cord Fos protein expression (Fig. 3C, Table 2).

4. Discussion

Previous laboratory studies have demonstrated that the intraperitoneal injection of inhalation anesthetics also leads to antinociceptive effects in mice [2,10,12]. According to pharmacokinetics and our pilot experiments, we confirmed that i.p. or s.c. injection of sevoflurane can achieve the same analgesic effect as inhalation.

In this experiment, the dosage of 2.5 ml/kg of emulsified sevoflurane was selected. Systemic administration of emulsified sevoflurane at this dosage did not impair motor performance in rats, but showed an obvious antinociceptive response against formalin injection. Moreover, intrathecal injection of L-Arg in conscious rats had no obvious effects on motor performance but significantly inhibited the antinociception of sevoflurane in the formalin test. Therefore, these results indicated that the reduction in the

Table 2
Fos expression in the spinal cord in rats ($n = 6$, $\bar{X} \pm S$).

Group	Entire section	Lam I–II	Lam III–IV	Lam V–VI	sLam VII–X
NS	11 ± 3	6 ± 3	3.1 ± 1.2	1.5 ± 1.0	0 ± 0
Sev	11 ± 3	5 ± 4	4.3 ± 1.5	1.4 ± 1.1	0 ± 0
F	222 ± 18	120 ± 13	57 ± 6	36 ± 6	25 ± 4
NS_{ip} + F	220 ± 20	122 ± 12	40 ± 7	37 ± 10	32 ± 6
NS_{it} + F	204 ± 25	85 ± 16	50 ± 12	45 ± 10	27 ± 7
Sev + F	126 ± 18*	65 ± 8*	24 ± 4*	20 ± 4*	18 ± 3*
L-Arg $_{it}$ + F	200 ± 26	95 ± 14	36 ± 9	38 ± 8	31 ± 7
NS_{it} + Sev + F	100 ± 12*	46 ± 10*	22 ± 5	27 ± 6*	14 ± 3*
L-Arg_{it} + Sev + F	145 ± 19*,#	70 ± 11*,#	44 ± 6#	19 ± 7*	12 ± 4*

* $P < 0.05$ compared with the Group NS_{ip} + F.
\# $P < 0.05$ compared with the Group NS_{it} + Sev + F.

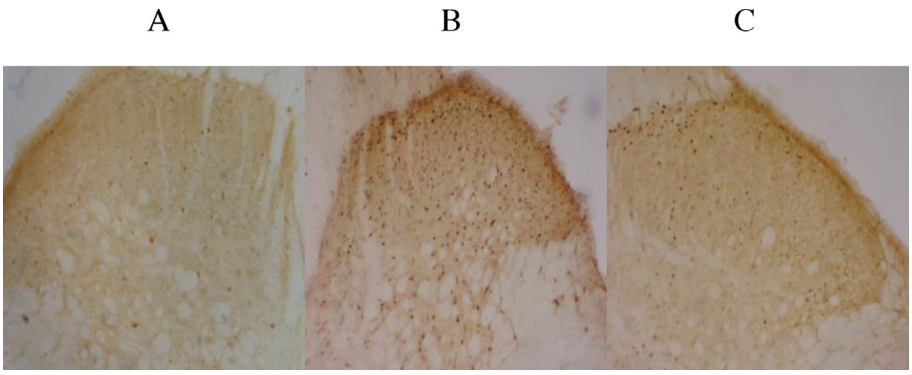

Fig. 3. A: Fos expression induced by formalin stimulation in the spinal cord in rats (Group F). B: Pretreatment with intrathecal NS had no effects on the suppression of i.p. sevoflurane on spinal Fos expression induced by formalin stimulation in rats (Group NSit + Sev + F). C: Pretreatment with intrathecal L-Arg_{it} 20 μg obviously attenuated the suppression of sevoflurane on formalin-induced spinal cord Fos protein expression ($P < 0.05$) (Group L-Arg_{it} + Sev + F) (original magnification: ×100).

formalin-induced paw licking response after sevoflurane injection was a result of antinociception but not an impairment of motor function.

Fos has only a low level of basal expression in the normal spinal cord tissue [15]. Peripheral stimulation can activate the Fos expression in the spinal cord. To a certain extent, the numbers of FLI neurons were directly associated with the stimulation intensity, and this has been used extensively to evaluate the analgesic efficacy of medicines [16–18]. In agreement with behavioral studies, intraperitoneal injection of sevoflurane caused a significant reduction in the amount of Fos-LI induced by formalin injection in the spinal cord, suggesting that sevoflurane produces antinociceptive effects partly at the spinal level.

It is well accepted that NO is an important biological messenger in the central nervous system, including in nociception transmission [19]. Feng found that NOS expression in hippocampal neurons was significantly decreased after sevoflurane exposure [20]. So we speculated that the expression of NO was involved in sevoflurane-induced antinociceptive effects. In our study, intrathecal pretreatment with NO precursor L-arginine alone neither affected the formalin-induced pain behavior nor spinal cord Fos expression, but inhibited sevoflurane-induced antinociceptive effects and its suppressive effects on Fos expression. This result indicates that spinal cord NO is involved in the antinociception of sevoflurane in rats.

The spinal cord is the first relay site in the transmission of nociceptive information from the periphery to the brain. Nociceptive signals are transmitted from the periphery by primary afferent fibers into the dorsal horn of the spinal cord. L-Arg the NO precursor, attenuated the reduction of sevoflurane in the amount of F-LI in laminae I–IV in the spinal cord, suggesting that laminae I–IV were related more closely with the antinociception of sevoflurane. Pretreatment of sevoflurane in our study did not completely inhibit Fos expression, suggesting that sevoflurane at that dosage could not completely suppress the nociceptive signal transmission at the spinal level.

There are some limitations relevant to the interpretation of the results of the present study. First, the number of rats in each group was small. Second, there is no objective indicator to evaluate the depth of anesthesia, so we could only judge and evaluate the depth of anesthesia indirectly by the movement ability of the rats. Third, although we observed and discussed the effect of different dosages of L-arginine on the antinociceptive effects of sevoflurane, we did not discuss the effects of the time of intrathecal injection of L-arginine on the antinociceptive effects of sevoflurane. Fourth, the results of this study can only indirectly speculate that spinal NO may be involved in antinociceptive effects of sevoflurane on pain induced by formalin in rats. Further studies are needed to find the direct evidence to see whether sevoflurane has analgesic effects via the spinal production of NO.

We found that intrathecal pretreatment with the NO precursor L-arginine significantly reduced the antinociceptive effects of sevoflurane in the formalin test. Furthermore, the systemic injection of sevoflurane significantly decreased the formalin-induced increases in Fos-LI labeled neurons in the spinal cord, which could be partly reversed by L-arginine. Based on these data, we concluded that the inhibition of spinal NO production may contribute to the antinociceptive effects of sevoflurane on formalin-induced pain in rats.

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References

[1] X.Q. Li, X.Z. Cao, J. Wang, B. Fang, W.F. Tan, H. Ma, Sevoflurane preconditioning ameliorates neuronal deficits by inhibiting microglial MMP-9 expression after spinal cord ischemia/reperfusion in rats, Mol. Brain 4 (2014) 69.
[2] Y.W. Wang, X.M. Deng, X.M. You, S.X. Liu, Z.Q. Zhao, Involvement of GABA and opioid peptide receptors in sevoflurane-induced antinociception in rat spinal cord, Acta Pharmacol. Sin. 26 (2005) 1045–1048.
[3] J.J. Ivanusic, The pattern of Fos expression in the spinal dorsal horn following acutenoxious mechanical stimulation of bone, Eur. J. Pain 12 (2008) 895–899.
[4] I.O. Lee, R. Yukhananov, D.G. Standaert, G. Crosby, NMDA-R1 antisense oligodeoxynucleotides modify formalin-induced nociception and spinal c-Fos expression in rat spinal cord, Pharmacol. Biochem. Behav. 79 (2004) 183–188.
[5] L.L. Bellavance, A.J. Beitz, Altered c-fos expression in the parabrachial nucleus in a rodent model of CFA-induced peripheral inflammation, J. Comp. Neurol. 366 (1996) 431–447.
[6] J. Wu, L. Fang, Q. Lin, W.D. Willis, Fos expression is induced by increased nitric oxide release in rat spinal cord dorsal horn, Neuroscience 96 (2000) 351–357.
[7] P. Hassanzadeh, A. Ahmadiani, Nitric oxide and c-Jun N-terminal kinase are involved in the development of dark neurons induced by inflammatory pain, Synapse 59 (2006) 101–106.
[8] F. Bulutcu, A. Dogrul, M.O. Güç, The involvement of nitric oxide in the analgesic effects of ketamine, Life Sci. 71 (2002) 841–853.
[9] M. Kakinohana, Protective effects of anesthetics on the spinal cord, Curr. Pharm. Des. 20 (2014) 5744–5750.
[10] J. Deng, C. Lei, Y. Chen, Z. Fang, Q. Yang, H. Zhang, M. Cai, L. Shi, H. Dong, L. Xiong, Neuroprotective gases – fantasy or reality for clinical use? Prog. Neurobiol. 115 (2014) 210–245.
[11] T.L. Yaksh, T.A. Rudy, Chronic catheterization of the spinal subarachnoid space, Physiol. Behav. 17 (1976) 1031–1036.
[12] Z.J. Ge, T.J. Dai, Y.M. Zeng, S.M. Duan, Effect of pretreatmnet with intrathecal prazosin on the antinociception of ketamine in rats, Chin. Pharmacol. Bull. 21 (2005) 427–431.
[13] A.R. Oliveira, H.M. Barros, Ultrasonic rat vocalizations during the formalin test: a measure of the affective dimension of pain? Anesthesia Analg. 102 (2006) 832–839.

[14] I. Long, R. Suppian, Z. Ismail, The effects of pre-emptive administration of ketamine and norBNI on pain behavior, c-Fos and prodynorphin protein expression in the rat spinal cord after formalin-induced pain is modulated by the DREAM protein, Kor. J. Pain 26 (2013) 255–264.
[15] Z. Khoshdel, M.A. Takhshid, A.A. Owji, Intrathecal amylin and salmon calcitonin affect formalin induced c-fos expression in the spinal cord of rats, Iran. J. Med. Sci. 39 (2014) 543–551.
[16] H. Fukagawa, T. Koyama, K. Fukuda, κ-Opioid receptor mediates the antinociceptive effect of nitrous oxide in mice, Br. J. Anaesthesia 2 (2014), pii: aeu254. [Epub ahead of print].
[17] T. Yang, L. Zhuang, A.M. Rei Fidalgo, E. Petrides, N. Terrando, X. Wu, R.D. Sanders, N.J. Robertson, M.R. Johnson, M. Maze, D. Ma, Xenon and sevoflurane provide analgesia during labor and fetal brain protection in a perinatal rat model of hypoxia-ischemia, PLoS 7 (2012) e37020.
[18] T. Swyers, D. Redford, D.F. Larson, Volatile anesthetic-induced preconditioning, Perfusion 29 (2014) 10–15.
[19] N. Toda, H. Toda, Y. Hatano, Nitric oxide: involvement in the effects of anesthetic agents, Anesthesiology 107 (2007) 822–842.
[20] X. Feng, J.J. Liu, X. Zhou, F.H. Song, X.Y. Yang, X.S. Chen, W.Q. Huang, L.H. Zhou, J.H. Ye, Single sevoflurane exposure decreases neuronal nitric oxide synthase levels in the hippocampus of developingrats, Br. J. Anaesthesia 109 (2012) 225–233.