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Effect of the Deficiency of Spinal PSD-95/SAP90 on the Minimum Alveolar Anesthetic Concentration of Isoflurane in Rats

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Background: **Spinal *N*-methyl-D-aspartate (NMDA) receptor activation has been demonstrated to play an important role in the processing of spinal nociceptive information and in the determination of the minimum alveolar anesthetic concentration (MAC) of inhalational anesthetics. Postsynaptic density-95 (PSD-95)/synapse-associated protein-90 (SAP90), a molecular scaffolding protein that binds and clusters the NMDA receptor perferentially at synapses, was implicated in NMDA-induced thermal hyperalgesia. The current study investigated the possible involvement of PSD-95/SAP90 in determining MAC for isoflurane anesthesia.**

Methods: **Sprague-Dawley rats were pretreated intrathecally with PSD-95/SAP90 antisense oligodeoxyribonucleotide (ODN), sense ODN, missense ODN, or saline every 24 h for 4 days. After initial baseline determination of the MAC, NMDA or saline was injected intrathecally. Ten minutes later, MAC measurement was repeated. The rats also were evaluated for the presence of locomotor dysfunction by intrathecal administration of NMDA or saline in the saline- and ODN-treated rats.**

Results: **In the groups treated with antisense ODNs, but not in those treated with sense or missense ODNs, there was a significant decrease in isoflurane MAC that was not accompanied by marked changes in either blood pressure or heart rate. In the saline-treated group, intrathecal NMDA caused an increase in isoflurane MAC. In contrast, in the antisense ODN–treated group, intrathecal NMDA did not produce a significant change in isoflurane MAC. An NMDA-induced increase in blood pressure but not heart rate was found in both saline- and antisense ODN–treated groups. Locomotor activity was not changed in any of the treated animals.**

Conclusion: **The results indicate not only a significant decrease in MAC for isoflurane but also an attenuation in the NMDA-induced increase in isoflurane MAC in the PSD-95/SAP90 antisense-treated animals, which suggests that PSD-95/SAP90 may mediate the role of the NMDA receptor in determining the MAC of inhalational anesthetics.**

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THE potency of anesthetic agents to inhibit the ability of a patient to respond with movement to painful stimuli has long been used as a test of anesthetic action. This potency, characterized by its ED_{50}, is widely known as the minimum alveolar concentration (MAC). Several lines of evidence have shown that spinal *N*-methyl-D-aspartate (NMDA) receptor activation might play a key role in the processing of nociceptive information[1–3] and in the determination of the MAC of inhalational anesthetics.[4–6] For example, the NMDA receptors are distributed mainly in the superficial laminae of the spinal cord.[7,8] Both repetitive C-fiber stimulation and direct application of glutamate or NMDA produce spinal neuronal sensitization and enhance responsiveness, which can be blocked by NMDA receptor antagonists.[2,9–11] Behavioral studies demonstrate that spinal administration of NMDA produces thermal hyperalgesia, caudally directed scratching and biting, and exaggerated responsiveness to light touch.[12–15] Moreover, antagonism of the spinal NMDA receptors produces antinociception in numerous animal models of pain[15–20] and reduction in the MAC of isoflurane.[4–6] However, the molecular mechanisms underlying these actions remain unknown.

The postsynaptic density (PSD), a highly organized cytoskeletal structure found adjacent to the postsynaptic membrane of excitatory synapses, is believed to play a role in the organization of receptors and related proteins involved in synaptic signaling.[21,22] A number of proteins enriched in the PSD have been characterized.[23,24] One of these proteins, PSD-95/synapse-associated protein-90 (SAP90), is an abundant scaffolding molecule that binds and clusters the NMDA receptor preferentially at synapses in the brain and spinal cord.[25–30] This raises the possibility that PSD-95/SAP90 might be involved in many physiologic and pathophysiologic actions triggered *via* the NMDA receptors and perhaps other receptors in the central nervous system. Indeed, suppression of PSD-95/SAP90 expression attenuated excitotoxicity produced *via* NMDA receptor activation in brain neurons.[31] The lack of PSD-95/SAP90 revealed an enhanced NMDA-dependent long-term potentiation and impaired learning.[32] Recently, we found that the deficiency of PSD-95/SAP90 in the spinal cord decreased NMDA-induced thermal hyperalgesia,[30] suggesting that PSD-95/SAP90 might be involved in spinal processing of nociceptive information. In the present study, we examined whether PSD-95/

SAP90 in the spinal cord was involved in determining the MAC of isoflurane.

Materials and Methods

The present study protocol was approved by the Animal Care Committee at the Johns Hopkins University. Male Sprague-Dawley rats (250–300 g) were housed individually in cages on a standard 12 h–12 h light–dark cycle. Water and food were available *ad libitum* until rats were transported to the laboratory approximately 1 h before the experiments. All experiments were performed during the same conditions.

Animal Preparation

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg). Long-term intrathecal catheters were inserted by passing a polyethylene-10 catheter through an incision in the atlanto-occipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar subarachinoid space using methods described previously.[33] The animals were allowed to recover for 5–7 days before experiments were initiated. Rats that showed neurologic deficits postoperatively were removed from the study.

To examine whether the deficiency of the expression of PSD-95/SAP90 affected the threshold for isoflurane anesthesia, we designed an antisense oligodeoxyribonucleotide (ODN) corresponding to the PSD-95–DLG–Z0-1 domain nucleotides 241–258 (5'-TGTGATCTCCTCATACTC-3') of rat *PSD-95/SAP90* mRNA, as well as the sense ODN and missense ODN (5'-AAGCCCTTGTTCCCATTT-3').[30] All of the ODNs were searched to exclude nonspecificity of the sense or antisense ODNs and to show that missense ODN did not match any confounding sequences in the GenBank database (GenBank accession number M96853). The ODNs were made and purified with the use of high-performance liquid chromatography (Integrated DNA Technologies, Inc., Coralville, IA). As described in previous work,[30] the ODNs were dissolved in saline before administration. The rats were injected intrathecally with saline (10 μl; control), antisense ODN (12.5, 25, 50 μg/10 μl), sense ODN (50 μg/10 μl), and missense ODN (50 μg/10 μl), respectively, followed by an injection of 10 μl saline to flush the catheter, every 24 h for 4 days.

Measurement of Minimum Alveolar Concentration

On the fifth day after saline or ODN injection, each rat was placed in a clear plastic cone and anesthetized with 5% isoflurane in oxygen for 3–5 min. After tracheostomy, the trachea of each animal was intubated with a 16-gauge polyethylene catheter. The inspired isoflurane concentration was reduced to 2%, and the animals breathed spontaneously until cannulation of a carotid artery and a jugular vein with polyethylene-50 tubing was accomplished. The isoflurane concentration was decreased further to 1.5%, and ventilation was controlled by an animal respirator adjusted according to the measurement of arterial blood gases to maintain normal partial pressure of oxygen (91–94 mmHg), partial pressure of carbon dioxide (33–41 mmHg), and pH (7.4–7.44). Electrocardiography and systolic and diastolic blood pressure were monitored. Rectal temperature was maintained between 36.5 and 37.5°C with a heating blanket and warming lights.

A polyethylene-10 catheter was introduced through and beyond the endotracheal tube until obstruction to passage was met and was then withdrawn 1 to 2 mm. For isoflurane MAC measurement, the polyethylene-10 catheter was connected to a parameter airway gas monitor. After stabilizing approximately 30 min, MAC was measured according to previously described methods[34] using a long hemostat clamped to the first ratchet lock on the tail for 1 min. The tail was always stimulated proximal to a previous test site. Gross movement of the head, extremities, or body was taken as a positive test result, whereas grimacing, swallowing, chewing, or tail flick were considered negative results. The isoflurane concentration was reduced in decrements of 0.12–0.15% until the negative response became positive, with 12–15-min equilibration allowed after changes in concentration.[35,36] The MAC was considered to be the concentration midway between the highest concentration that permitted movement in response to the stimulus and the lowest concentration that prevented movement. Finally, intrathecal polyethylene-10 catheter position from each animal was confirmed.

In some saline-treated rats, after initial baseline MAC determination, NMDA (Sigma, St. Louis, MO) at the dose of 1.25 μg[14] or saline was injected intrathecally in a volume of 10 μl saline, followed by an injection of 10 μl saline to flush the catheter. Fresh NMDA solution was prepared for each experiment. An isoflurane concentration was chosen at which movement did not occur in the last negative response before the positive test response. At this isoflurane concentration, 10 min after the intrathecal injection of NMDA, the animals were tested again for reactivity to tail clamp. The concentration of isoflurane was increased, and response to tail clamp was checked every 12–15 min thereafter until a negative response was achieved. In some antisense ODN (50 μg)-treated rats, after initial MAC determination, NMDA or saline was also administered intrathecally. The MAC for isoflurane was again determined after the aforementioned procedures.

Tests of Locomotor Function

The effects of ODNs on locomotor function were examined using the following methods.[37] The animals

Table 1. Effects of Antisense (AS), Sense (SE), and Missense (SE) Oligodeoxyribonucleotides and Saline on Isoflurane MAC, Blood Pressure (BP), and Heart Rate

	Saline (n = 14)	12.5 μg AS (n = 6)	25 μg AS (n = 6)	50 μg AS (n = 6)	50 μg SE (n = 6)	50 μg MS (n = 6)
MAC	1.16 ± 0.08	1.15 ± 0.18	0.98 ± 0.14*	0.72 ± 0.05*	1.15 ± 0.21	1.13 ± 0.15
BP (mmHg)						
Systolic	119.86 ± 10.58	127.58 ± 11.72	122.75 ± 10.81	129.58 ± 11.73	126.67 ± 10.40	121.33 ± 15.84
Diastolic	106.36 ± 7.78	112.58 ± 7.14	105.83 ± 7.89	112.50 ± 11.20	105.58 ± 13.07	105.75 ± 11.40
Heart rate (beats/min)	513.00 ± 40.78	534.80 ± 29.13	541.20 ± 16.70	514.20 ± 62.20	529.60 ± 22.61	524.70 ± 44.90

* $P < 0.01$ *versus* saline-treated (control) group.

MAC = minimum alveolar concentration.

were organized randomly into six groups: control (saline), 12.5 μg antisense ODN, 25 μg antisense ODN, 50 μg antisense ODN, 50 μg sense ODN, and 50 μg missense ODN. The rats were pretreated with ODNs or saline in the manner described above. On the fifth day, 10 μl saline was injected intrathecally for each rat. In some saline or antisense ODN (50 μg)-treated rats, fresh NMDA solution (1.25 μg/10 μl) was injected intrathecally. The following tests were performed with the experimenter blind to which group was treated with the agents:

1. Placing reflex: The rat was held with the hind limbs slightly lower than the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. The experimenter recorded whether the hind paws were placed on the table surface reflexively.
2. Grasping reflex: The rat was placed on a wire grid and the experimenter recorded whether the hind paws grasped the wire on contact.
3. Righting reflex: The rat was placed on its back on a flat surface and the experimenter noted whether it immediately assumed the normal upright position.

Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex shown in five trials. In addition, the rat general behaviors including spontaneous activity were observed.

Statistical Analysis

The MAC data were assessed statistically by an analysis of variance. Intergroup differences were analyzed using the Newman-Keuls test. Locomotor data were assessed by a rank sum test. All data are reported as the mean ± SD. Significance was set at $P < 0.05$.

Results

The value for isoflurane MAC in the control (saline-treated) group was 1.16 ± 0.08, which is consistent with that in previous studies.[34,38] In the groups treated with the antisense ODN at the doses of 12.5, 25, and 50 μg, the isoflurane MAC values were decreased from isoflurane control MAC by 1%, 18% ($P < 0.01$), and 44% ($P < 0.01$), respectively (table 1). In contrast, intrathecal administration of sense ODN at the dose of 50 μg or missense ODN at the dose of 50 μg did not significantly change the value for isoflurane MAC compared with the control group (table 1). No untoward effects were observed in any of the treated animals, including the antisense groups. In the ODN-treated groups, there was no significant change in either blood pressure or heart rate compared with the control group before the tail clamp (table 1). Control baseline blood pressure was 119.86 ± 10.58 mmHg systolic and 106.36 ± 7.78 mmHg diastolic, and control baseline heart rate was 513.00 ± 40.28 beats/min.

In the saline-treated group, intrathecal NMDA at a dose of 1.25 μg caused an increase from isoflurane control MAC by 15% ($P < 0.01$; fig. 1). The NMDA-induced change in isoflurane MAC was accompanied by a significant increase in systolic and diastolic blood pressures (135.70 ± 3.38 mmHg and 118.30 ± 7.81 mmHg, respectively; $P < 0.05$ *vs.* control) but not in heart rate (529.20 ± 55.20 beats/min; $P > 0.05$ *vs.* control). However, in the group pretreated with 50 μg antisense ODN, intrathecal administration of 1.25 μg NMDA did not result in a significant increase in isoflurane MAC compared with the group treated with 50 μg antisense ODN

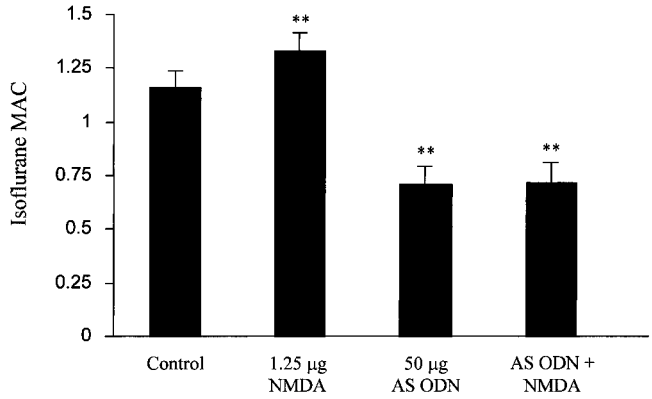

Fig. 1. Effect of intrathecal administration of *N*-methyl-D-aspartate on isoflurane minimum alveolar concentration (MAC) in the saline- and PSD-95/SAP90 antisense oligodeoxyribonucleotide (AS ODN)–treated groups. Data are presented as mean ± SD, n = 5 animals for each group, except n = 14 for the saline-treated (control) group. **Significantly different from control ($P < 0.01$).

Table 2. Mean (SD) Changes in Locomotor Test

Agents	Placing	Grasping	Righting
Saline	5 (0)	5 (0)	5 (0)
12.5 μg AS	5 (0)	5 (0)	5 (0)
25 μg AS	5 (0)	5 (0)	5 (0)
50 μg AS	4.83 (0.41)	4.67 (0.52)	4.83 (0.41)
50 μg SE	5 (0)	5 (0)	5 (0)
50 μg MS	5 (0)	5 (0)	5 (0)
Saline + 1.25 μg NMDA	5 (0)	5 (0)	5 (0)
50 μg AS + 1.25 μg NMDA	4.83 (0.41)	4.83 (0.41)	4.83 (0.41)

N = 6, five trials.

AS = antisense; SE = sense; MS = missense; NMDA = *N*-methyl-D-aspartate.

alone ($P > 0.05$; fig. 1). Interestingly, in the group pretreated with 50 μg antisense ODN, intrathecal NMDA at a dose of 1.25 μg still produced a significant increase in systolic and diastolic blood pressures (138.00 ± 5.77 mmHg and 117.00 ± 6.35 mmHg, respectively; $P < 0.05$ *vs.* the group that received 50 μg antisense ODN alone) but not in heart rate (553 ± 17 beats/min; $P > 0.05$ *vs.* control).

As shown in table 2, ODNs with or without NMDA at the doses used in the current study did not produce significant effects on locomotor function. Convulsions and hypermobility were not observed in any of the treated animals, including the antisense ODN groups. In addition, there was no significant difference in general behaviors, including spontaneous activity between the control and the ODN-treated groups.

Discussion

The current study indicates that pretreatment of PSD-95/SAP90 antisense ODN, but not sense or missense ODN, produced a remarkable reduction in isoflurane MAC. This was not accompanied by changes in either blood pressure or heart rate. Furthermore, it was found that the PSD-95/SAP90 antisense ODN blocked NMDA-induced increase in isoflurane MAC in the current work. Our results suggest that the deficiency of PSD-95/SAP90 expression may produce anesthetic and analgesic actions at the spinal cord level and that PSD-95/SAP90 might mediate the role of the NMDA receptor in determining the MAC of inhalational anesthetics.

Antisense ODNs have been widely used as research tools, and even as drugs in clinical trials. Antisense ODNs inhibit protein expression by the mechanisms of (1) steric blockade of ribosomal subunit attachment to mRNA at the 5' cap site; (2) interference with proper mRNA splicing through antisense binding to splice donor or splice acceptor sites; and (3) RNase-H–mediated degradation of hybridized mRNA.[39] The proper design and controls of experiments are critical in demonstrating a true antisense effect. In the current study, the specificity of intrathecal treatment with PSD-95/SAP90 antisense ODN has been shown. First, we designed the standard controls of equivalent sense sequence and missense ODNs. Neither had any effect on the isoflurane MAC. This indicates the specificity of the inhibition observed with the antisense ODN. Second, all of the ODNs had been searched to exclude nonspecificity of the sense or antisense ODNs and to show that missense ODN did not match any confounding sequences in the GenBank database. Moreover, our previous results have demonstrated that antisense ODNs only suppressed the expression of PSD-95/SAP90 but not the expression of NMDA receptor subunits NR2A and 2B, neuronal nitric oxide synthase, or SAP102 (a protein that is closely related to the targeted protein) in the spinal cord.[30] These results suggest that the effects observed after treatment with the PSD-95/SAP90 antisense ODN are unlikely to be explained by changes in the expression of other proteins. Finally, the antisense ODN at the doses used in the present study only affected isoflurane MAC without untoward effects in any of the treated animals, including the antisense groups. Considering these several lines of evidence, we believe that the effects we have described may be caused by a direct and selective interference of the antisense ODN with mRNA transcripts of *PSD-95/SAP90* and to the blockade of protein production *via* binding to the nucleotides of *PSD-95/SAP90* mRNA.

The regional expression and function of PSD-95/SAP90 in the mammalian brain have been investigated using a variety of experimental approaches.[25–29] PSD-95/SAP90 immunoreactivity was found mainly in cortex, hippocampus, and cerebellum.[40–42] In brain neurons, suppression of PSD-95/SAP90 expression that selectively disrupted physical linkage of the NMDA receptor with neuronal nitric oxide synthase has been demonstrated to attenuate excitotoxicity and Ca^{2+}-activated nitric oxide production *via* NMDA receptor activation.[31] Mice carrying a targeted mutation in the *PSD-95/SAP90* gene showed an enhanced NMDA-dependent long-term potentiation and impaired learning.[32] Recently, we found that the mRNA and protein of *PSD-95/SAP90* also were enriched in the spinal cord and selectively distributed in the superficial dorsal horn, where PSD-95/SAP90 expression overlapped with that of the NMDA receptor.[7,8] In the spinal neurons, PSD-95/SAP90 interacted with the NMDA receptor subunits 2A and 2B.[30] Behavioral studies showed that intrathecal administration of antisense ODN for PSD-95/SAP90 significantly attenuated facilitation of the tail-flick reflex triggered through the NMDA receptor activation.[30] This evidence indicates that activation of the NMDA receptor in spinal neuronal sensitization results in association of the NMDA receptor with PSD-95/SAP90 and that PSD-95/SAP90 is required for the spinal mechanisms of hyperalgesia. This suggests that PSD-95/

SAP90 may be involved in the processing of pain and that the deficiency of PSD-95/SAP90 may produce analgesic action at the spinal cord level. Such an action is consistent with our current report of the effect of the deficiency of PSD-95/SAP90 on MAC. In the current experiments, we chose doses of antisense ODN that did not cause motor and general behavioral dysfunction when administered intrathecally in rats. We believe that the effect of suppression of spinal PSD-95/SAP90 expression that resulted in the reduction in MAC in the current study may be a result of effects on analgesia alone. However, PSD-95/SAP90 has been demonstrated to be involved in the mechanisms of long-term potentiation and learning.[32] Whether the antisense ODN for PSD-95/SAP90 produces a sedative effect or decrease consciousness is unknown, although an effect of antisense ODN on righting reflex was not observed in the current study. The possibility of these actions of the antisense ODN in the central nervous system could not be ruled out from the current study because the intrathecal antisense effect had a segmental nature.

A role for the NMDA receptors in determining the MAC of inhalational anesthetics is suggested by the fact that the systemic or intrathecal administration of NMDA antagonists significantly reduces the MAC of isoflurane in rats, which is completely reversed to control level by intrathecal administration of NMDA.[4–6] The current study further indicated that intrathecal administration of NMDA increased the MAC of isoflurane in saline-treated rats. Interestingly, in antisense ODN–treated rats, intrathecal injection of NMDA did not affect the MAC of isoflurane. Our previous study showed that PSD-95/SAP90 completely overlapped with the NMDA receptor subunits 2A and 2B in spinal superficial dorsal horn.[30] Furthermore, with the use of co-immunoprecipitation, it was found that the PSD-95/SAP90 antibody was able to immunoprecipate not only PSD-95/SAP90 but also NR2A and 2B *in vivo*.[30] These findings demonstrate that PSD-95/SAP90 interacts with NR2A and 2B in the spinal cord *in vivo*. Combined with the current results, it is suggested that PSD-95/SAP90 is essential for the actions of the NMDA receptor in determining the MAC of inhalational anesthetics. The present finding may provide novel insights into the mechanisms of anesthesia and potentially novel sites for therapeutic intervention.

In our experiments, no significant hemodynamic effects were observed in the ODN-treated animals during isoflurane anesthesia. However, intrathecal administration of NMDA resulted in a significant increase in systolic and diastolic blood pressure during isoflurane anesthesia in both the saline- and antisense ODN–treated groups. It has been demonstrated that sympathetic preganglionic neurons located in the intermediate nucleus of the spinal cord are integral elements in the neural pathway linking the central nervous system to sympathetic nerves supplying the heart and blood vessels.[43,44] The effects of NMDA on blood pressure may be a result of the involvement of the NMDA receptor in regulation of sympathetic output at the spinal cord level. In immunohistochemical studies, glutamate and its receptors were found in the intermediolateral nucleus of the thoracic spinal cord.[45,46] Intrathecal administration of NMDA at the T10 level increased arterial pressure. This action was blocked by NMDA receptor antagonists.[47,48] It is likely that NMDA, administered intrathecally at the lumbar level in this study, activates spinal sympathetic activity in the intermediolateral nucleus and produces the increase in blood pressure. We found that the antisense ODN had no effect on the hemodynamics or on the NMDA-induced increase in blood pressure, a finding that is consistent with our previous observation that PSD-95/SAP90 was absent or present at extremely low levels in the intermediolateral nucleus of the spinal cord.[30] It could be that the second message signaling pathways in the somatic and the sympathetic nervous systems are different with respect to the NMDA receptor. Hong and Henry[47] and West and Huang[48] reported that microinjection of NMDA into the intermediolateral nucleus at the spinal T2 level or intrathecal injection of NMDA at the T10 level produced an increase in heart rate. Interestingly, the effect of NMDA on heart rate was not observed in either saline- or antisense ODN–treated groups in the current study. The reason for this discrepancy between the previous and the current studies is not clear and may be a result of a difference in anesthetic agents (isoflurane in the current study *vs.* urethane, chloral hydrate, and sodium pentobarbitone). It is interesting to note that intrathecal administration of the NMDA receptor antagonist, (±)-2-amino-5-phosphonovaleric acid, produced a dose-related decrease in arterial pressure but not in heart rate.[47,49] These data suggest that there is a tonic activation of the NMDA receptor in the spinal sympathetic pathway to the vessels but not to the heart.

In conclusion, we showed that there was not only a significant decrease in MAC for isoflurane but also an attenuation in the newly observed NMDA-induced increase in isoflurane MAC in the PSD-95/SAP90 antisense-treated animals. The binding of PSD-95/SAP90 to the NMDA receptor preferentially at synapses in the spinal cord and brain suggests that PSD-95/SAP90 may mediate the role of the NMDA receptor in determining the MAC of inhalational anesthetics. Our results may provide novel insights into the mechanisms of anesthesia and indicate new targets for drug development.

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