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Central noradrenergic mediation of nitrous oxide-induced analgesia in rats

Purpose: Although several studies have demonstrated that both supra opiate receptors and spinal α_2 adrenoceptors play a mediating role in nitrous oxide (N_2O) analgesia, controversy still exists. The present study was undertaken to evaluate further the involvement of noradrenergic (NA) neuronal activity in N_2O analgesia by investigating tail-flick latency and supra- and spinal NA levels in rats.

Methods: In an analgesia study, effect of N_2O 75% and its modification were evaluated using the tail-flick test in male Wistar rats. Results were expressed as % maximum possible effect (MPE). Modification of N_2O analgesia was examined in rats pretreated with either the α_2 receptor agonist, clonidine (CLO: $150 \mu\text{g}\cdot\text{kg}^{-1}$, ip), α_2 receptor antagonist, idazoxone (IDZ: $100 \mu\text{g}\cdot\text{kg}^{-1}$, iv) by lesioning the locus coeruleus (LC) seven days before exposure to N_2O , or naloxone ($5\text{mg}\cdot\text{kg}^{-1}$, iv). Also, in a NAergic neuronal transmission study, the changes in NA content at LC and spinal cord were determined using HPLC-ECD.

Results: Nitrous oxide produced analgesia, % MPE increased to a maximum of 78% at 30 min, thereafter declining to 38% at 120 min. Clonidine potentiated the analgesic effect of N_2O at 120 min (80%). The analgesic effect of N_2O was attenuated by IDZ or by LC lesioning. However, naloxone, in a dose sufficient to block morphine-induced analgesia, had no effect. With N_2O exposure, NA content was decreased by 52% in the LC and by 20% at spinal cord. With morphine, NA content did not differ from the control group.

Conclusion: The data suggest that N_2O -induced analgesia is principally mediated by activation of the descending inhibitory NAergic system and/or increased NA release at spinal cord which may lead to presynaptic inhibition of primary afferent neurotransmitter release and hyperpolarize the dorsal horn neurons by α_2 receptors.

Objectif : Bien que plusieurs études aient démontré que les suprarécepteurs opiacés et les récepteurs α_2 -adrénergiques rachidiens jouent un rôle de médiation dans l'analgésie au protoxyde d'azote (N_2O), la controverse persiste. La présente étude a été entreprise pour évaluer plus amplement la participation de l'activité neuronale noradrénergique (NA) dans l'analgésie avec N_2O en examinant le temps de latence des coups de queue et les niveaux noradrénergiques des suprarécepteurs et des récepteurs rachidiens.

Méthode : Dans une étude sur l'analgésie, l'effet du N_2O 75 % et des modifications qu'il produit ont été évalués en utilisant la technique du coup de queue chez des rats mâles Wistar. Les résultats ont été exprimés en termes de pourcentage de l'effet maximal possible (EMP). La modification de l'analgésie au N_2O a été étudiée chez des rats prétraités avec l'agoniste du récepteur α_2 , la clonidine (CLO : $150 \mu\text{g}\cdot\text{kg}^{-1}$, ip), l'antagoniste du récepteur α_2 , l'idazoxone (IDZ : $100 \mu\text{g}\cdot\text{kg}^{-1}$, iv) en sectionnant le locus coeruleus (LC) sept jours avant l'exposition au N_2O , ou avec du naloxone ($5\text{mg}\cdot\text{kg}^{-1}$, iv). Également, dans l'étude de la transmission neuronale noradrénergique, les changements de contenu de noradrénaline dans le LC et dans la moelle épinière ont été déterminés en utilisant la CLHP (chromatographie liquide à haute performance) et le DCE (détecteur à capture d'électrons).

Résultats : Lors de l'analgésie produite par le protoxyde d'azote, le pourcentage de l'EMP a augmenté pour atteindre 78 % à 30 min, pour décliner par la suite à 38 % à 120 min. La clonidine a potentialisé l'effet analgésique du N_2O à 120 min (80%). L'effet analgésique du N_2O a été diminué par l'IDZ ou par la section du LC. Cependant, le naloxone, en dose suffisante pour bloquer l'analgésie induite par la morphine, n'a pas eu d'effet. Avec l'exposition au N_2O , le contenu de noradrénaline a diminué de 52 % dans le LC et de 20 % à la moelle épinière. Avec la morphine, le contenu de noradrénaline n'a pas été différent de celui du groupe témoin.

Conclusion : Les données suggèrent que l'analgésie induite par le N_2O bénéficie principalement de la médiation de l'activation du système noradrénergique inhibiteur descendant et/ou de la libération accrue de noradrénaline au niveau de la moelle épinière qui peut mener à une inhibition présynaptique de la libération du neurotransmetteur afférent primaire et à l'hyperpolarisation des neurones de la corne supérieure par les récepteurs α_2 .

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NITROUS oxide (N_2O) has a potent antinociceptive action but the mechanism is uncertain. Kitahata *et al.*¹ demonstrated electrophysiologically that N_2O inhibited the spontaneous firing of wide dynamic range (WDR) neurons of the spinal cord in decerebrate and spinal cats, suggesting that the antinociceptive action of N_2O may involve inhibition of neuronal transmission at the spinal level.

Further investigations suggested that N_2O may interact with opioid receptors at both spinal and supraspinal levels.²⁻⁵ Berkowitz *et al.*² reported, in rats, that N_2O had a potent antinociceptive effect to chemical and heat stimuli, and that this effect was antagonized by naloxone. In addition, Zuniga *et al.*⁵ showed that the analgesic effect of N_2O was attenuated by lesioning of periaqueductal gray matter (PAG) in rats and that N_2O increased content of β -endorphin of PAG.⁴ However, the importance of their opioid-related mechanisms is uncertain because of the extremely large amount of naloxone needed to antagonize N_2O analgesia.²

It has recently been demonstrated that noradrenergic (NA) nerve tracts originating from the locus coeruleus (LC) or A7 neurons are innervated by presynaptic terminals of C fibres and that the WDR of the spinal cord, the descending NAergic inhibitory system, and α_2 receptors are densely localized in the dorsal horn neurons of the spinal cord,¹⁰ suggesting that spinal α_2 receptors may modulate primary afferent input. Intrathecal (*it*) injection of NA or the α_2 receptor agonist, clonidine, produced potent analgesic effects assessed by acute,¹¹ hyperalgesic¹² or neuropathic pain states.¹³ These effects were counteracted by the simultaneous delivery of an α_2 receptor antagonist.¹⁴ Since N_2O stimulates the central NAergic system, the analgesic action of N_2O might be mediated by the effect on this system. Also, Guo *et al.*¹⁵ has suggested that both supra-spinal opioid and spinal α_2 adrenoceptors mediate the antinociceptive response to N_2O in rats.

Accordingly, the present study was undertaken to evaluate further the effect of N_2O on NAergic neuronal activity at supra-spinal and spinal levels in relation to NAergic neuronal transmission. In addition, the effects of N_2O were compared with those of morphine.

Materials and methods

The experimental protocols were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine and Kyushu Dental College, and carried out according to the Guidances for Animal Experimentation at Yamaguchi University School of Medicine, and The Law (No, 105) and Notification (No, 6) of the Japanese Government.

Animals

Male Wistar rats, weighing 250 to 350 g, were housed with lights on from 7:00 to 19:00 and allowed free access to food and water.

Protocol for evaluation of analgesia

Under halothane, 2-3%, anaesthesia, a PE-50 catheter was inserted into the femoral vein for drug administration. Then, anaesthesia was discontinued and the rats were placed individually in specially designed plastic boxes (7 × 7 × 7 cm) for at least 30 min for stabilization. Rats were randomly assigned to one of seven groups:

- i) Control group (n = 8): Rats breathed N_2 75% in O_2 .
- ii) No pretreatment (N_2O alone) (n = 8): Rats received saline, *iv*, five minutes before N_2O exposure.
- iii) Clonidine group (n = 8): 150 $\mu\text{g}\cdot\text{kg}^{-1}$ clonidine *ip* was given 30 min before N_2O exposure.
- iv) Idazoxane group (n = 8): 100 $\mu\text{g}\cdot\text{kg}^{-1}$ idazoxane *iv* was given at the start of N_2O exposure.
- v) LC lesion group (n = 3): An LC lesion was made by electrical coagulation seven days before N_2O exposure. The lesion was confirmed by postmortem microscopical examination.
- vi) Morphine group (n = 5): 5 $\text{mg}\cdot\text{kg}^{-1}$ morphine, *iv* was given five minutes before N_2 exposure.
- vii) Naloxone group (n = 10): 5 $\text{mg}\cdot\text{kg}^{-1}$ naloxone, *iv* was given either by simultaneous delivery with morphine five minutes before N_2 exposure (n = 5) or at the start of exposure to N_2O (n = 5).

Throughout the experiments, rectal temperature was kept at $37.2 \pm 0.2^\circ\text{C}$ by a heating lamp. After the start of exposure to N_2O (or N_2 in the control group) the analgesic effect was measured by means of the tail-flick testing over 120 min. Measurements of tail-flick latency were repeated at 15, 30, 60, 90, and 120 min after the start of 75% N_2O (or N_2) exposure or morphine injection.

To assess the analgesic effect of N_2O , tail-flick latency (Analgesia meter, MK-330, Muromachi Kikai, Tokyo, Japan) was measured as previously described.¹⁶ Before N_2O (or N_2) exposure or morphine injection, three to five consecutive control measurements were performed; the median value of these measurements was taken as the baseline. The median value of three to five consecutive measurements at each time was calculated. The potency of the analgesic effect was evaluated by calculating the maximal possible effect (MPE) as follows:

TFT - TFT'

$$\% \text{ MPE} = \frac{\text{CO} - \text{TFT}}{\text{TFT} - \text{TFT}'} \times 100$$

CO - TFT'

where, TFT' is the baseline tail-flick time (4-6 sec), TFT is the experimental tail-flick time, and CO is the cut-off time (=10 sec).

Protocol for evaluation of NAergic neuronal transmission

Rats were assigned to one of three groups:

- i) Control (N₂ 75%, n = 5)
- ii) N₂O (75%, n = 6)
- iii) Morphine (5 mg·kg⁻¹, *iv*, n = 5)

The changes in NA content were measured in the LC bilaterally and the dorsal part of spinal cord. After 120 min of N₂O (or N₂) exposure or morphine injection, the rats were killed by decapitation. The brain and the spinal cord were quickly removed according to rat atlas and placed in an ice-cooled box at 4°C and were frozen with isopentane/freon (-40°C). The tissue block was weighed and homogenized using an ultrasonic sonicator. After centrifugation at 10,000 rpm for 20 min at -20°C, 50 µl of the supernatant (approximately 0.3 ml) were used for determination of NA content using high performance liquid chromatography with an electrochemical detector (HPLC-ECD; 600 mv). The sensitivity of the assay for NA content was 5-10 ng·mg⁻¹. The assay was linear over the concentrations of 100 ng to 2000 ng·mg⁻¹.

FIGURE 1 Time course of maximal possible effect (MPE) of nitrous oxide (75%) in rats: (nitrogen exposure (n = 8); •: nitrous oxide exposure (n = 8), Values are mean ± SEM, * P < 0.05 compared with control.

FIGURE 2 Modification of the analgesic effect of nitrous oxide with various treatments assessed after 30 min and 120 min exposure.

P < 0.05 compared with control # or with nitrous oxide alone*

FIGURE 3 Modification of nitrous oxide or morphine analgesia by naloxone assessed after 30 min and 120 min exposure to nitrous oxide or morphine administration.

P < 0.05 compared with control* or with nitrous oxide group #

TABLE Effect of N₂O exposure on NA content in CNS (ng·mg⁻¹).

Group	Spinal cord (I-II)	LC
Control (n = 5)	549 ± 57	1430 ± 144
N ₂ O (n = 6)	442 ± 68*	680 ± 81*
Morphine (n = 5)	411 ± 104	985 ± 128

Values indicate mean ± SEM

* ($P < 0.05$) compared with Control group

Statistical analysis

Results are presented as means ± SEM. For evaluation of both analgesic effect by % MPE and the NA content among groups, significant differences were tested by one-way analysis of variance (ANOVA) followed by the least significance difference (LSD) method for multi-comparison of means. $P < 0.05$ was considered to be significant.

Results

Analgesic effect of N₂O and its modification

The time course of the analgesic effect as expressed by % MPE in the rats exposed to N₂O alone and to N₂ with morphine is shown in Figure 1. The rats in the control group consistently demonstrated no change and stable % MPE values throughout the 120 min observation period. In the N₂O alone group, % MPE increased, reaching a maximum of 78% at 30 min, thereafter % MPE declined to 38% at 120 min ($P < 0.05$). Since the analgesic effect of N₂O was attenuated with time, the modification with various pretreatment was assessed at 30 min and 120 min. These two time points could be representative as interpretation over 120 min. The intravenous injection of clonidine did not affect the analgesic effect of N₂O at 30 min exposure (% MPE, 70%), but potentiated N₂O-induced analgesia at 120 min (% MPE, 80%; $P < 0.05$). By contrast, intravenous pretreatment with IDZ and LC lesioning attenuated the analgesic effect of N₂O (Figure 2).

After intravenous pretreatment with naloxone the % MPE increase produced with morphine was blocked. However, the % MPE increase with N₂O was only partially blocked and this did not reach statistical difference (Figure 3).

Noradrenergic neuronal transmission

In the N₂O group, NA content was greater in the LC than in the dorsal horn of its spinal cord. Nitrous oxide decreased NA content by 52% in the LC and by 20% in

the dorsal part of spinal cord ($P < 0.05$). With morphine, NA content did not change, compared with control, in any region (Table).

Discussion

The principal findings of the present study are: i) the antinociceptive effect of N₂O was inhibited by the α_2 receptor antagonist idazoxane and by LC lesioning, ii) N₂O decreased the NA content at spinal cord. This suggests that supra-spinal NAergic activation plays a pivotal role in the antinociceptive response to N₂O in the spinal cord which confirmed the findings of a recent report.¹⁵

Mediation of N₂O induced analgesia by the enkephalinergic system has been postulated previously but there remains some controversy. Guo *et al.*¹⁵ demonstrated that N₂O analgesia may be mediated by activation of a descending inhibitory NAergic pathway that may, in turn, be activated by opioid receptors in the PAG region. They suggested that both supra-spinal opioid and spinal α_2 adrenoceptors played a mediating role in the antinociceptive effect of N₂O in rats. The present study provides additional evidence that NA neuronal activity, in particular the analgesic effect, was inhibited by both α_2 receptor antagonist and LC lesioning, and activation of NAergic neuronal transmission that decreased NA content in the spinal cord which reflects increased release¹⁷ and leads to activation of α_2 receptor localized at C fibre terminal and dorsal horn neurons. This means that descending NAergic neurons originating from the LC to substantia gelatinosa of spinal cord, the site of C fibre input, are involved in the mechanism of N₂O analgesia. However, although naloxone was given systematically, it is difficult to determine whether its site of action of opioid activity concerned with N₂O analgesia is supra-spinal or spinal level.

N₂O analgesia and its modification: involvement of NAergic neuronal activity in CNS

The present study showed that N₂O analgesia, evaluated by the tail-flick test, was enhanced by the systemic delivery of an α_2 receptor agonist and was inhibited by α_2 receptor antagonists or by LC lesioning. Thus, we suggest that N₂O analgesia may be mediated by activating the central NAergic system.

Tail-flick testing is a reflection of the multi-synaptic spinal reflex via polymodal C fibres with high-threshold nociceptive stimuli. Therefore, the present results indicate that the analgesic effect of N₂O is mediated by modulation of primary afferent sensory transmission via C fibres as a presynaptic event, and/or postsynaptic event. Thus, inhibition of the primary afferent sensory transmission by N₂O appears to be mediated by activation of the central NAergic system.

Based on the present results i) LC lesioning attenuated the analgesic effect of N₂O and, ii) N₂O markedly decreased the NA content at the LC. According to Lisopraweki *et al.*,¹⁷ accumulation or depletion of neurotransmitter in a specific region is due to decrease or increase, respectively, in release from the neuron terminal. Thus, the increased NA release from the LC neuron (activation of NA neuron at LC), suggests that at least part of the antinociceptive effect of N₂O involves stimulation of the LC neuron leading to activation of the descending NA inhibitory pathway and increased release of NA at spinal level. Lesioning of the LC did not abolish the analgesic effect of N₂O, suggesting that N₂O produced an analgesic effect, at least in part, by other mechanisms which might include supra-spinal enkephalinergic neurons.¹⁵

Recent concepts of analgesic mechanisms suggest that one of the pivotal regulatory systems is situated in the dorsal horn of the spinal cord. For example, morphine may inhibit substance P (sP) release from primary afferents nerve terminal in the dorsal horn, and it may also activate descending inhibitory neurons originating from the PAG and reticular nuclei^{7, 8} via descending NAergic and serotonergic pathways. The NAergic descending system originating from the LC modulates synaptic transmission of primary afferent C or Aδ fibres at pre and post synaptic neurons of the spinal cord via the activation of α₂ receptor and by inhibiting release of sP.^{18,19}

Using an electrophysiological method, the analgesic effect of N₂O results from inhibiting spontaneous firing in the dorsal horn of spinal cord in cats.^{1,20} A number of pharmacological studies subsequently indicated that N₂O analgesia may be mediated by μ-opioid receptors. Berkowitz *et al.*² reported that, in mice, N₂O has a potent antinociceptive effect to chemical and heat stimuli in a dose-dependent manner, and that this effect was antagonized by naloxone. In addition, Zuniga *et al.*⁵ showed that the analgesic effect of N₂O was attenuated by PAG lesioning in rats. This effect was correlated with the inhaled N₂O concentration and the β-endorphin content of hypothalamus or PAG. However, a large amount of naloxone was needed to antagonize N₂O analgesia.² In addition, a recent report indicated that N₂O analgesia was more likely to be mediated by κ-opioid receptors than by μ-opioid receptors.²¹ We could not show any involvement of opioid neuronal activity on N₂O analgesia using naloxone at a dose that antagonized morphine analgesia. Taken together, involvement of opiate receptors in mediating with N₂O analgesia remains controversial.

In the present study, to correlate behavioural changes with synaptic events, we measured NA content

in both the LC and superficial layer of the dorsal horn of the spinal cord. The decrease in NA content in LC and spinal cord support the concept that N₂O produced an antinociceptive effect by activating both supra-spinal and spinal NAergic neurons.

NAergic nerve fibres are widely distributed throughout the central nervous system including the substantia gelatinosa of the dorsal horn, that originated from the LC.¹⁰ Microscopical observation has demonstrated that, in the dorsal horn of the spinal cord, α₂ receptors are densely localized at pre and postsynaptic sites of C and A fibres.¹⁰ Furthermore, α₂ receptor activation exerts potent inhibition on nociception through the mechanisms of i) increased Gi / K⁺ conductance-adenylate cyclase system resulting in hyperpolarization of WDR neurons²⁰ and, ii) inhibition of presynaptic neurotransmitter release from C fibres coupled with Go / N-type Ca channels.²² NAergic neuronal fibres located in the spinal cord originate mostly from the LC.¹⁰ Electrical stimulation of the LC caused an antinociceptive effect to the dorsal horn neurons of the spinal cord.²³ Therefore, the NAergic neuronal system originating from the LC is thought to be a principle component of the descending inhibitory pathway. In fact, in spinal transected rats, clonidine suppresses veratridine-evoked release of substance P from slices of rat spinal cord²² and also clonidine-evoked prolongation of tail-flick latency.¹¹ Thus, it appears that clonidine produces a potent anti-nociceptive effect by activation of α₂ receptors in the dorsal horn of the spinal cord. In addition, Ishikawa²⁴ has demonstrated that N₂O exposure increased sP content at spinal cord, suggesting N₂O decreases sP release from afferent nerve terminal of the small fibre and that is consistent with attenuation of sP release by activation of α₂ receptors in the dorsal horn of the spinal cord. These earlier data together with the present results provide substantial support for the involvement of NAergic neuronal activity in N₂O analgesia.

Comparison with morphine analgesia

In this study, we confirmed that morphine produced marked analgesia and that this analgesic effect was blocked by systemic delivery of naloxone. Also, the effect of NAergic synapse transmission by morphine was examined to compare it with the action of N₂O. Our results showed that with morphine, NA content did not alter. The potent antinociceptive effect of morphine is mediated by activation of supra-spinal neurons located in the PAG or paraventricular cellular reticular nucleus.^{7,25} Therefore, morphine may produce analgesia by interacting not only by activation of spinal cord containing interneurons, but also with the descending inhibitory system, NAergic and/or sero-

toninergic pathway via an activation of opioid receptors at the supraspinal level. In the present study, NA content with morphine was decreased although not statistically significant, suggesting that increases in NA release at the spinal level are related to activation of interneurons of spinal cord and/or LC activation. We cannot exclude the possibility that morphine produces activation of NAergic inhibitory system.

Based on these results, it appears that the mechanisms for N₂O analgesia may be mediated by activation of the NAergic system at supra-spinal and spinal cord levels which appears to be different from that of opioid (morphine) analgesia. However, the present study needs confirmation that N₂O analgesia is mediated by activation of opiate receptors localized at periaqueductal grey (PAG) and dorsal horn of spinal cord.

In summary, it is suggested that N₂O-induced analgesia is mediated by activation of both supra-spinal and spinal NAergic neurons, that this leads to presynaptic inhibition of primary afferent neurotransmitter release as well as hyperpolarization of dorsal horn neurons in the spinal cord.

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