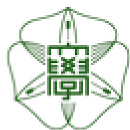
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Influence of nitrous oxide on granule cell migration in the dentate gyrus of the neonatal rat

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ABSTRACT

For several decades, the neurotoxicities of anesthetics to the developing brain have been reported by many researchers focusing on various phenomena such as apoptosis, neurodegeneration, electrophysiological aberrations, and behavioral abnormalities. According to these reports, signals via N-methyl-D-aspartate receptors (NMDA-r) and/or γ-aminobutyric acid type A receptors ($GABA_A$-r) are implicated in the anesthetic neurotoxicity. On the other hand, during brain development, NMDA-r and $GABA_A$-r are also recognized to play primary roles in neural cell migration. Therefore, anesthetics exposed in this period may influence the neural cell migration of neonates, and increase the number of hilar ectopic granule cells, which are reported to be a cause of continuous neurological deficits. To examine this hypothesis, we investigated immunohistochemically granule cell distribution in the hippocampal dentate gyrus of Wistar/ST rats after nitrous oxide (N_2O) exposure. At postnatal day (P) 6, 5-bromo-2'-deoxyuridine (BrdU) was administered to label newly generated cells. Then, rats were divided into groups ($n = 6$ each group), exposed to 50% N_2O at P7, and evaluated at P21. As a result, we found that ectopic ratios (ratio of hilar/total granule cells generated at P6) were decreased in rats at P21 compared with those at P7, and increased in N_2O exposed rats for over 120 min compared with the other groups. These results suggest that 50% N_2O exposure for over 120 min increases the ratios of ectopic granule cells in the rat dentate gyrus.

Almost all anesthetics—ketamine (15, 54), midazolam, nitrous oxide (N_2O) (16), propofol (6), isoflurane (25), and sevoflurane (39)—have been suggested to cause apoptosis and neurodegeneration of immature neurons in neonatal mammals. However, the apoptosis and neurodegeneration are not insufficient to fully explain the mechanisms of postgrowth disabilities such as learning deficits (50), behavioral disorders (18), and developmental abnormalities (8), because it has been reported that there was no correlation between the degree of apoptotic neurodegeneration and the persistent behavioral deficits (11). In addition, the number and density of neurons have also been known to recover with sufficient passage of time after anesthetic exposure (24). Therefore, unknown mechanisms of anesthetic neurotoxicity other than apoptosis and neurodegeneration can be inferred.

In this regard, focusing on neonatal neural development of the hippocampus, it has been reported that granule cell precursors in the dentate gyrus were generated in not only the subgranular zone but also the dentate hilus (1). The immature granule cells generated in the dentate hilus gradually migrate to the granule cell layer (1), and then matured into granule cells by about three weeks after birth (21, 35). This neuronal precursor migration is thought to be essential for proper development of the neonatal central nervous systems (13, 44), and considered to

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be regulated by GABA (2, 28) and/or NMDA (14, 20) signaling.

On the other hand, granule cells which failed to migrate properly and became trapped in the dentate hilus were called hilar ectopic granule cells (hEGCs) (40). They have been reported to increase by various stimuli such as febrile seizures and drug administration (21, 51), and additionally, these abnormally migrated hEGCs were considered to construct aberrant networks with other neurons (7), provide a cause of electrographic seizures (37), and induce adult epilepsy (21).

Considering these facts, we developed a hypothesis that one of the contributing factors to explain the neurotoxicities of anesthetics is abnormally increased hEGCs due to anesthetic exposure. Therefore, to investigate the effect of N_2O, which has been used for clinical anesthesia in the world, we observed the distribution of newly generated granule cells, and evaluated the ectopic ratios (ratio of hilar/total newly generated granule cells) using an immunohistochemical method.

MATERIALS AND METHODS

Materials. These experiments were approved by the Committee for Animal Research of the Hokkaido University Graduate School of Medicine. Male Wistar/ST rats were supplied by Shizuoka Laboratory Animal Center (Hamamatsu, Japan). They were housed in a room maintained at 22°C to 25°C with a 12-hour light and dark cycle (lights on 06 : 00 to 18 : 00, lights off 18 : 00 to 6 : 00), and were given free access to food and water. To avoid litter variability, six rat pups of each litter were divided into the various groups. These rats were treated with scrupulous adherence to the Guidelines for the Care and Use of Laboratory Animals of the Hokkaido University Graduate School of Medicine which is implemented in relation to NIH Guide for the Care and Use of Laboratory Animals.

5-Bromo-2'-deoxyuridine (BrdU) injection. At postnatal day 6, all rat pups were subcutaneously administered 300 mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO; dispensed at 15 mg/mL in a physiological saline), which is a thymidine analog that incorporates DNA of dividing cells during the S-phase of the cell cycle (33). As such, BrdU is widely used for birth dating and monitoring cell proliferation (46, 48). The bioactivity of this dose of BrdU continues for less than four hours after administration (5), so we could label only neoplastic cells that were generated exclusively in the last minutes before our treatments at P7.

Nitrous Oxide. N_2O is a volatile anesthetic having almost pure NMDA receptor (NMDA-r) antagonistic ability (17, 31), that is commonly used in general anesthesia particularly for infants because of its ability to produce a quick and stable state of unconsciousness with minimal respiratory depression in comparison to other available agents (12, 38). This anesthetic was appropriately administrated to neonatal rats as explained below.

Gas exposure. Rats were exposed to warm, humidified gas in an acrylic chamber that had two glove ports to treat animals. To maintain the environment, the total gas flow was maintained at over 3 liters per min, and the concentrations of N_2O and O_2 were continuously monitored using an anesthetic gas monitor (5250RGM; Detex-Ohmeda GE Healthcare, Chalfont St. Giles, UK). In general, anesthetics are considered to decrease body temperature because of their potent effects on the redistribution of blood and metabolic capacity (29). Therefore, the body temperatures of rats were measured with a Thermofocus (Tecnimed, Tokyo, Japan) and maintained at 35.5°C to 36.5°C by using a heater with a fan installed on the bottom of the chamber. After gas exposure, the rats were housed in their original cages.

Tissue preparation. For immunohistochemical examinations, rat tissues were fixed at P7 or P21. Rats were deeply anesthetized with halothane and then transcardially perfused with heparinized 0.1 M phosphate-buffered saline (PBS) followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. After this, the brains were removed and immersed overnight in the same fixative for postfixation followed by immersion in 20% sucrose in the phosphate buffer at 4°C until they sank, after which we repeated the same process with 30% sucrose. Then, the brains were embedded in OCT compound (Tissue-Tek O.C.T.; Sakura Finetek, Japan), frozen thoroughly between −20 and −25°C, and cut with a cryostat (CM1850; Leica Biosystems, Wetzlar, Germany) into 40-μm-thick coronal sections containing the dentate gyrus. Every third (P7), or twelfth (P21) section was picked up and placed on a glass slide.

Double-labeled immunofluorescence staining. The sections were rinsed three times with PBS. To stain BrdU which is incorporated into neoplastic cells, DNA denaturation was performed with 30-minute

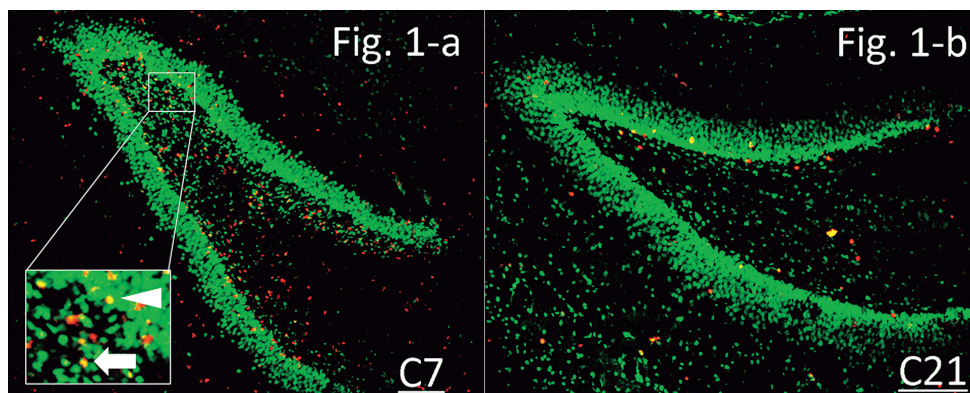

Fig. 1 Confocal microscopic stack images of rat dentate gyrus at postnatal day 7 (left) and postnatal day 21 (right). BrdU-positive nuclei were labeled in red and Prox1-positive nuclei were labeled in green. Double labeled cells observed in yellow were newly generated granule cells. At P21, we counted the hEGCs (arrow) and normally migrating granule cells in the granule cell layer (arrow head), and calculated the ratios of hEGCs to total newly generated granule cells.

incubation in 1N HCl at 45°C followed by three rinses for five minutes each in PBS at room temperature. Then the sections were blocked with 3% donkey serum in PBS for 60 min at room temperature followed by reaction with a mixture of an anti-BrdU antibody (47) (1 : 1,000, mouse monoclonal; Sigma-Aldrich, St. Louis, MO) and an antibody against the prospero homeobox protein 1 (Prox1) which was used as a marker of granule cells (22, 36) (1 : 500, rabbit polyclonal; Covance, Princeton, NJ) in PBS containing 3% donkey serum and 0.3% Triton-X for 24–48 h at 4°C. After washing with PBS, the sections were incubated with a mixture of AlexaFluoro 488 (1 : 500, donkey anti-rabbit; Invitrogen, Carlsbad, CA) and AlexaFluoro 594 (1 : 500, donkey anti-mouse; Invitrogen) in PBS containing 0.3% Triton-X for two hours at room temperature in the dark. After that, the sections were washed three times for five minutes each with PBS followed by one-minute rinsing in distilled water and air-dried.

Finally, these processed sections were coverslipped with VectaShield mounting medium (Vector Laboratories, Burlingame, CA).

Confocal microscopy. The localization of BrdU and Prox1 colabeled cells was assessed using a laser scanning confocal microscope (FV1000; Olympus, Tokyo, Japan). Each section was scanned at 1-µm steps under a x-10 and x-20 objective lenses and collected as Z-direction images followed by stacking and reconstruction with 17 images using FLUOVIEW software (version 3.1a; Olympus).

Experiment 1. The difference in distribution of hEGCs between P7 and P21. First, to investigate the normal migration of immature granule cells in the dentate gyrus, rat pups were divided into two groups, as follows: (1) C7 group ($n = 6$), fixed at P7 with no treatment, (2) C21 group (n = 6), fixed at P21 with no treatment.

Experiment 2. N_2O exposure. Second, to investigate the effect of N_2O on immature granule cells in the dentate gyrus, rat pups were divided into three additional groups of 6 rats each and exposed to 50% N_2O with 50% O_2 for (1) 30 min (N30 group), (2) 120 min (N120 group), and (3) 240 min (N240 group) at P7. Tissues from each group were fixed at P21 and compared with those of C21 groups, which were prepared in Experiment 1.

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software, Inc., San Diego, CA). We used one-way ANOVA with Tukey's multiple comparison tests or Student's paired *t* test in statistical analysis. All data were described as means ± SEM, and probability values (*P*) of less than 0.05 were considered statistically significant.

RESULTS

Experiment 1. The difference in distribution of hEGCs between C7 and C21

Fig. 1-a and 1-b showed the representative confocal microscopic stack images of rat dentate gyrus at C7 and C21, respectively. Sections of the dentate gyrus were double-labeled using antibodies against 5-bromo-2'-deoxyuridine (BrdU) and homeobox prospero-like protein (Prox1). BrdU-positive nuclei were

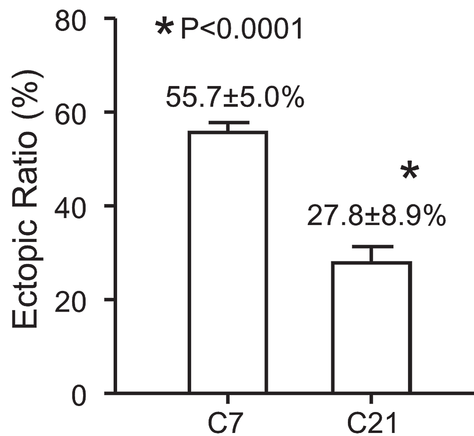

Fig. 2 The ectopic ratios of the C7 and C21 groups. The ectopic ratios of the C7 and C21 groups were 55.7 ± 5.0% and 27.8 ± 8.9%, respectively. The ectopic ratio of C7 was significantly higher than that of C21 group (Student's *t* test, $P < 0.0001$).

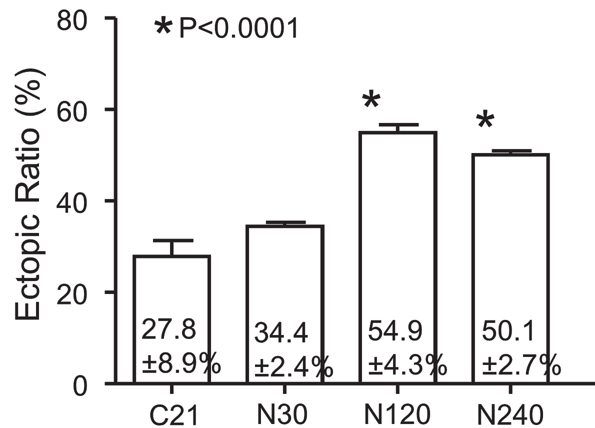

Fig. 3 The ectopic ratios of C21, N30, N120, and N240 groups. The ectopic ratios of the C21, N30, N120, and N240 groups were 27.8 ± 8.9%, 34.4 ± 2.4%, 54.9 ± 4.3%, 50.1 ± 2.3%, respectively. Statistical analysis revealed that there was no significant difference between the C21 and N30 groups. On the other hand, the ectopic ratios of both N120 and N240 groups were significantly higher than that of the C21 group (one-way ANOVA with post hoc Tukey, $F_{(3, 21)} = 36.3$, $P < 0.0001$).

labeled in red and Prox1-positive nuclei were labeled in green. Double-positive cells which were seen yellow were newly generated granule cells at P6. Confocal microscopic stack images were shuffled and assigned to three researchers blinded to the experimental conditions, and all the double labeled cells were counted manually for the hilus (arrow) and the granule cell layer (arrow head) in each rat, the dentate hilus was defined as that inside the line connecting the edge of the CA3 pyramidal cell layer, the edge of the upper blade of the granule cell layer, the inner border line of the granule cell layer, and the edge of the lower blade of the granule cell layer. Cells located within 10 μm of the inner border of the granule cell layer were considered to be localized in the granule cell layer because this area was the subgranular zone.

Finally, we calculated the ectopic ratios (ratios of hilar/total colabeled granule cells) of the C7 and C21 groups, which were 55.7 ± 5.0% and 27.8 ± 8.9%, respectively (Fig. 2). The ectopic ratio of the C7 groups was significantly higher than that of the C21 groups (Student's paired *t* test, $P < 0.0001$). Considering the fact that many of immature granule cells generated in the dentate hilus were being reduced in number and migrated gradually toward the granule cell layer in about three weeks (21, 35), these results seemed to be plausible.

Experiment 2. N_2O exposure

The ectopic ratios of the N30, N120, and N240 groups were 34.2 ± 2.4%, 54.9 ± 4.3%, and 50.1 ± 2.3%, respectively. Compared with the C21 group (in Experiment 1), there were significant increases of the ectopic ratios in the N120 and N240 groups (one-way ANOVA with the post hoc Tukey, $F_{(3, 21)} = 36.3$, $P < 0.001$) (Fig. 3). These results indicate that (1) sufficient exposure to anesthetics, at least for 120 min, may be required for N_2O to disturb the normal migration of immature granule cells, and (2) exposure to N_2O for over 120 min may not have additional effects for this disturbance.

In addition, there was no significant difference between the N30 and C21 groups. This result suggested that possible complications caused by 50% N_2O exposure, namely cardiovascular disorders, hypoxia or hypercapnia, had little effect on granule cell migration in neonatal rats.

DISCUSSION

The present study revealed that exposure to 50% N_2O for over 120 min significantly increased the ectopic ratios of newly generated granule cells in the hippocampus of neonatal rats.

During the early postnatal days, neurogenesis is observed in both the subgranular zone and the dentate hilus (1). In the subgranular zone, neurogenesis occurs continuously throughout life (45), whereas, the hilar neurogenesis peaks P5 and P7 (35, 43) and it concludes by P20. Most precursors of granule cells generated in the dentate hilus are considered to migrate toward the granule cell layer, maturing into

granule cells (1), and contribute to memory processing for a prolonged period (19). However, a small number of hilar granule cells are observed in the normal hippocampus, with the ratios of hilar granule cells in a state of nature being 0.1 to 0.2% of the total granule cell population (49) ; approximately 1,000–2,000 granule cells are thought to be developed in the hilus per neonatal rat hippocampus (30). Though the function of these normal hilar granule cells has not been elucidated in detail, some studies have suggested that their function was very similar to that of matured granule cells in the granule cell layer (41, 42), therefore, the normal hilar granule cells were thought to be simply added to the population of granule cells residing in granule cell layer (40).

On the other hand, various stimuli, including febrile seizures (21), drug-induced seizures (53), genetic properties (32), and some kinds of signaling via $GABA_A$ receptor (2) or NMDA receptor (13) have been reported to increase the proportion of hEGCs. In addition, these abnormally increased hEGCs have been thought to establish aberrant synaptic connections with other neurons (40–42), generate abnormal GABA signaling (51, 52), and finally provide a cause of postgrowth neurological dysfunctions such as temporal epilepsy (21, 32).

In the Experiment 1, we had conducted an immunohistochemical analysis for neonatal rat pups to examine the natural course of migration and the reliability of this analysis, and found the ectopic ratios of the no-treatment control groups were approximately 50% at P7, and 30% at P21 (Figs. 1, 2). Though we have not conducted time-lapse observation in this study, Koyama *et al*. had investigated the localization of P5-generated hilar granule cells with time-lapse observation of BrdU-labeled cells, and found brisk migration of P5-generated hilar granule cells toward the granule cell layer. Therefore, we concluded that the decrease with duration of the ectopic ratios was also caused by the natural migration of hilar-generated granule cells toward the granule cell layer.

The Experiment 2 had be conducted to examine the influence of N_2O exposure on the migration of newly generated precursor granule cells, and we discovered that 50% N_2O exposure in neonatal period for over 120 min raised the increase of the ectopic ratios of granule cells in the postgrowth dentate gyrus. As we mentioned before, it has been reported that N_2O had NMDA receptor antagonistic activity (17), and signaling via NMDA receptor was essential for neuronal cell migration as well as the other neuronal growth processes such as neuronal differentiation, synaptogenesis, and elimination of synapses (3, 4, 27) during the brain development period. Consistent with our results, Komuro *et al*., using slice preparations of the developing mouse cerebellum (20), revealed that blockade of NMDA receptor curtailed neuronal cell migration, and enhancement of NMDA receptor activity increased the rate of cell movement. Collectively, our result indicated the possibility that a part of neurotoxicities of anesthetics could be explained by the disturbance of migration caused by anesthetics which had NMDA antagonistic property.

On the other hand, though neurotoxicity of anesthetics has been thought to be caused by excessive GABAergic signaling and subsequent hyperpolarization of immature granule cells (21), our preliminary data showed that the single administration of pentobarbital, which has $GABA_A$ agonistic property, had not significant effect on the migration of immature granule cells. In order to interpret this result, we propose a hypothesis that N_2O stimulated NMDA receptors expressing on GABAergic interneurons (34) and upregulated them (10, 23) to induce subsequent continuous NMDA signaling (23). This change may enlarge the size of GABAergic terminals in GABAergic interneurons and enhance GABA release (9), resulting in disturbance of the migration of immature granule cells, due to excessive GABAergic signaling.

The role of NMDA receptor in hippocampal granule cell migration is very complicated and not fully clarified (26). Additionally, we have not interpreted the interactions between immature granule cells and GABAergic interneurons. Further studies to identify the mechanism of anesthetic neurotoxicity are necessary.

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