

Inhibition of NR2B-containing NMDA receptors during nitrogen narcosis

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Key words

Brain; Hippocampus; Hyperbaric medicine; Neuron; NMDA receptor

Abstract

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Introduction: When humans breathe compressed air or N₂-O₂ mixtures at three to four atmospheres pressure, they will experience nitrogen narcosis that may possibly lead to a diving accident, but the underlying mechanisms remain unclear.

Methods: Mice were exposed to 1.6 MPa breathing a N₂-O₂ mixture adjusted to deliver an inspired PO₂ of 32–42 kPa. The electroencephalogram (EEG) and forced swimming test were used to evaluate the narcotic effect of nitrogen. Neuronal activity was observed via c-Fos expression in cortex and hippocampus tissue after decompressing to the surface. To further investigate underlying molecular mechanisms, we incubated cultured hippocampal neurons with various NMDA concentrations, and measured expression of NMDA receptors and its down-stream signal with or without 1.6 MPa N₂-O₂ exposure.

Results: Both the frequency of the EEG and the drowning time using the forced swimming test were significantly decreased during exposure to 1.6 MPa N₂-O₂ ($P < 0.001$). Additionally, in cultured hippocampal neurons, the increased levels of phosphorylated NR2B and cAMP-response element binding protein (CREB) induced by NMDA stimulation were significantly inhibited by exposure to 1.6 MPa N₂-O₂.

Conclusions: Our findings indicated that NR2B-containing NMDA receptors were inhibited during nitrogen narcosis.

Introduction

When human beings are exposed to hyperbaric inert gases e.g., nitrogen, argon and xenon, the central nervous system (CNS) will be inhibited due to their narcotic potency. Nitrogen narcosis has been recognized since the earliest studies of human and animal physiological responses to diving conditions, and includes disturbances in motor and locomotor coordination, hallucinations, sedation, and cognitive disruptions, often leading to diving accidents.^{1,2}

Franks and Lieb have proved that the inhibition of the N-methyl-D-aspartate receptor (NMDAR) is likely the critical mechanism in the anaesthetic and analgesic effects of xenon gas.³ Thus, we supposed that nitrogen, as another inert gas, may also act on NMDARs leading to narcosis. Glutamate plays a major role in the neurotransmission of excitatory signals via long axonal projections of neurons in the CNS and acts on diverse receptors including amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA), NMDAR, and kainate receptors. Some studies have revealed that selective blockade of excitatory glutamatergic neurotransmission in the CNS can induce general anaesthesia with unconsciousness, analgesia, and immobility.⁴ NMDARs are an important subtype of

glutamate receptors and may be targets of anaesthetics.⁵ NMDARs, composed of GluN1, GluN2 (GluN2A-D), and GluN3 (GluN3A-B) subunits, are expressed ubiquitously and abundantly throughout the human CNS and play a key role in regulating glutamatergic synaptic transmission. NMDAR channels require two GluN1 subunits and two GluN2 subunits or GluN3, and their functional properties are determined by the constitutive GluN2 subunits (GluN2A-D) and/or GluN3 (GluN3A-B).⁶ Among these subunits, NR2B is the most abundant in the CNS and it seems a molecular target of anaesthesia, thus we hypothesized that hyperbaric nitrogen might also (at least partly) act on this site.

Inhaled anaesthetics such as xenon and nitrous oxide have been shown to inhibit NMDAR. In electrophysiological studies, ketamine, nitrous oxide, and xenon are potent inhibitors of NMDA-activated currents.⁷ Ligand-gated ion channels on neurons have been all cited as target proteins for inert gas including nitrogen in animal models.^{8–12} It remains unclear whether they share a similar mechanism between general anaesthesia and nitrogen narcosis, therefore the present study was designed to investigate the changes in NMDAR in the development of nitrogen narcosis.^{3,7}

Methods

ANIMALS

Male adult (6–9 weeks of age) and pregnant C57BL/6j mice were provided by the Experimental Animal Center of Nantong University (Institutional license: SYXK(SU)-2012-0030). All animal use protocols were approved by the Institutional Animal Care and Use Committee of Nantong University (approval number 20140901-001). All efforts were made to minimize the number of animals used and their suffering. Behaviors relating to food and water intake, fecal character, hair color, mobility, and body weight were monitored daily. At the end of the experimental period, the mice were anaesthetized using isoflurane and killed by cervical dislocation under general anaesthesia. In total, fifty-five adult mice were randomly divided into two groups: a control group ($n = 24$) and a 1.6 MPa N_2 - O_2 mixture exposure group ($n = 31$). Mice were housed in group cages with 4–6 animals per cage at 23°C with food and water available *ad libitum* and maintained in a room with a 12/12 h light/dark cycle.

HYPERBARIC EXPOSURE

Hyperbaric exposure was performed as described in our previous experiment.¹³ Briefly, compressed air was introduced to the chamber (Wuhu Diving Equipment Factory, Anhui, China) at a rate of 100 kPa·min⁻¹ up to 200 kPa (additional pressure), then pressure was further increased to 1.6 MPa by addition of pure nitrogen (Nantong Tianyuan Gas Co. Ltd, Jiangsu, China) at 200 kPa·min⁻¹. Mice were maintained at 1.6 MPa for an hour, followed by five hours decompressing to atmospheric pressure. The concentrations of oxygen and carbon dioxide were monitored in real time by SDA monitors (Analox, North Yorkshire, England). Oxygen was added to maintain the inspired PO_2 between 32–42 kPa and this was increased to 50 kPa during decompression. Carbon dioxide levels were not allowed to exceed 1 kPa and CO_2 was removed using soda lime absorbent. To avoid the stress-induced changes involved in the hyperbaric exposure, mice in the control group were placed in the chamber at normobaric pressure only breathing air.

RECORDING OF EEG

Fifteen mice were anaesthetized with isoflurane and placed on a heating pad, and then two bipolar stainless steel electrodes were planted into the forehead (2 mm posterior to the bregma, 2 mm lateral from the midline, and 1 mm deep beneath the skull). The ground electrode was attached to prefrontal skin as described by Wisor.¹⁴ After surgery, mice were housed alone with free access to food and water. Digital EEG monitoring (RM6240BD, Chengdu Instruments, Chengdu, China) to evaluate the narcotic potency of nitrogen was performed when the electrodes were completely fixed at one week after the operation. During EEG recording,

the acquisition frequency was 1 kHz, the scan speed was 200 ms·div⁻¹, and the sensitivity was 1 μ V. Band width of the EEG signal recording was set as 0.5–30 Hz. Finally, the mean frequency of the EEG was analyzed using RM6240 3.0 software: alpha rhythm 8–12 Hz and beta rhythm 13–30 Hz were defined as fast waves; delta rhythm 1–3 Hz and theta waves 4–7 Hz were defined as slow waves.

FORCED SWIMMING TEST

A cylinder (30 cm height $\times$ 20 cm diameter) filled with 15 cm height of water (23 $\pm$ 2°C) was placed in the hyperbaric chamber, and the mouse was put in the cylinder before compression. The drowning time, defined as losing the ability to keep the head above the water, was scored during the hyperbaric N_2 - O_2 mixture exposure. A total of 30 mice were measured in this test.

PRIMARY NEURON CULTURE AND NMDA STIMULATION

Briefly, the hippocampus of an embryonic (E17–18) mouse was isolated, cut into pieces, and digested with 0.125% trypsin at 37°C for 12 min. The digested brain tissues were mildly triturated to a single-cell suspension, and plated at a density of 70,000 cells·cm⁻² on to coverslips or dishes coated with poly-L-lysine. The day of plating was counted as day-in-vitro (DIV) 0. The feeding medium containing neurobasal media (Thermo Fisher Scientific, Waltham, USA), B-27 neural culture reagent (Thermo Fisher Scientific, Waltham, USA), 0.5 mM L-glutamine (Thermo Fisher Scientific, Waltham, USA), 100 units·ml⁻¹ penicillin and 0.1 mg·ml⁻¹ streptomycin (Thermo Fisher Scientific, Waltham, USA), was changed by one-half every three days. Cultures were maintained in humidified air containing 5% CO_2 at 37°C. On DIV 11–12, the culture media was changed to the buffer solution containing 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.42), 10 mM D-Glucose, 0.8 mM $MgCl_2$, and the cells were exposed to 1.6 MPa N_2 - O_2 for an hour and decompressed to the surface as quickly as possible. To achieve global activation of NMDAR, neurons were stimulated with NMDA (Sigma-Aldrich, St. Louis, Missouri, USA) at different concentrations when the pressure reached 1.6 MPa.

CELL VIABILITY ASSAY

After decompression to atmospheric pressure, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (5 mg·ml⁻¹ in phosphate-buffered saline, PBS) was added to each culture well. After incubating at 37°C for four hours, the medium was removed, dimethylsulfoxide was added to each well, and the cells were incubated for another thirty minutes at 37°C. Finally, the absorbance was read at 570 nm using SN209941 multi-mode microplate readers (Bio-Tek, Winooski, USA). Data were normalized to 100% control values.

WESTERN BLOT ANALYSIS

After decompressing to the surface, mice were immediately killed by decapitation under isoflurane anesthesia, their brain tissues were quickly harvested, and carefully dissected into hippocampus and cortex on ice, and then cold tissue lysis buffer was added in. In cultured cells, cell lysis buffer was added into cultures at the end of the hyperbaric exposure, and then the chamber pressure was rapidly decreased at a rate of $0.1 \text{ MPa} \cdot \text{s}^{-1}$. The homogenate was ultrasonicated in an ice bath and then centrifuged for 10 min at 12,000 g at 4°C . The concentration of supernatant protein was tested using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, USA). An equal amount of protein ($10\text{--}40 \mu\text{g}$ as optimal for each antibody) for each sample was loaded into 8–12% Bis-Tris gel for electrophoresis. Polyvinylidene fluoride membranes with transferred proteins were blocked with 5% milk in tris buffered saline plus 0.1% Tween-20 for one hour and then with the following primary antibodies overnight at 4°C : glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, Darmstadt, Germany); rabbit anti-cleaved caspase-3 polyclonal antibody 1:1000 (Cell Signaling Technology (CST), Danvers, USA); mouse anti- β -actin 1:8000 (Sigma-Aldrich Co., St. Louis, USA); rabbit anti-AMPA Receptor 1 (GluA1) (CST, Danvers, USA); and rabbit anti-Phospho-AGluA1 (Ser845) (CST, Danvers, USA), phospho-NR2B (CST, Danvers, USA), and total NR2B (CST, Danvers, USA). After several washes in Tris-buffered saline, secondary IRDye 800 CW goat anti-mouse or rabbit 1:10000 (Li-COR, Lincoln, USA) was incubated for two hours at room temperature, and the immunoreactivities were captured using a fluorescence scanner (Odyssey Lix, Li-COR, Lincoln, USA). Semi-quantitative evaluation of protein levels was performed by densitometric scanning using Image-Pro® Plus 5.1 software (Media Cybernetics, Bethesda, USA).

IMMUNOFLUORESCENT STAINING

Cultured hippocampal neurons were washed three times with 0.01 M PBS, fixed with 4% paraformaldehyde, antigen-repaired in 0.1 M glycine for 10 minutes, permeabilized in pre-cooling methanol at -20°C for eight minutes, blocked in 10% bovine serum albumin for one hour at room temperature, and then incubated at 4°C overnight with anti-rabbit phospho-CREB (cAMP-response element binding protein) (CST, Danvers, USA). This was followed by incubation with Goat anti-Rabbit 1:200 (Sigma-Aldrich Co., St. Louis, USA) for one hour. After washing with PBS, cultured cells were captured under the same laser intensity settings using confocal microscopy (TCS SP8, Leica, Wetzlar, Germany).

STATISTICAL ANALYSIS

All the variables were tested for normal distribution using

Shapiro-Wilk test with SPSS 17.0 software (IBM, USA). Normally distributed data were presented as the mean (SD) and analyzed with an independent sample *t*-test or one-way analysis of variance (ANOVA) with the LSD post hoc test. Non-normal data were analyzed with the Mann-Whitney U test or Kruskal-Wallis test.

Results

HYPERBARIC $\text{N}_2\text{-O}_2$ EXPOSURE EFFECTS ON THE EEG AND MOTOR FUNCTION

The frequency of the EEG in the control group was 10.17 (1.6 SD) Hz while it was significantly decreased to 4.56 (1.01) Hz during the hyperbaric exposure (Figure 1A–B, $P < 0.001$ vs control group). Additionally, mice breathing $\text{N}_2\text{-O}_2$ at 1.6 MPa drowned more quickly than those at atmospheric pressure (Figure 1C, $P < 0.001$ vs. control group).

HYPERBARIC $\text{N}_2\text{-O}_2$ EXPOSURE AND C-FOS EXPRESSION

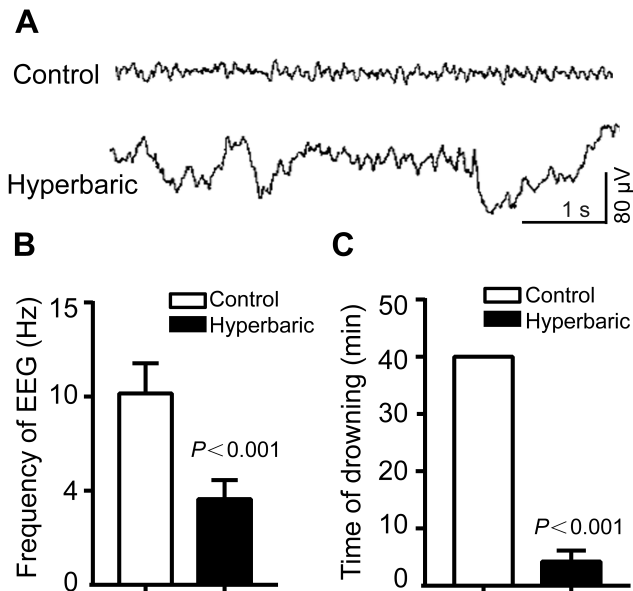
Neuronal activity assessed via cortical and hippocampal c-Fos expression was not significantly altered after hyperbaric $\text{N}_2\text{-O}_2$ exposure (Figure 2A–B, $P > 0.05$ vs. control group).

EFFECT OF NMDA INCUBATION ON CELL VIABILITY AND CLEAVED CASPASE-3 EXPRESSION IN CULTURED HIPPOCAMPAL NEURONS WITH OR WITHOUT $\text{N}_2\text{-O}_2$ EXPOSURE

In vivo, time is required for decompression to atmospheric pressure and changes in the behavioral performance and the physiological and metabolic functions of mice induced by hyperbaric $\text{N}_2\text{-O}_2$ exposure will recover gradually. Cultured hippocampal neurons were thus chosen for further investigation of the potential influences of hyperbaric exposure because only a short decompression time is needed. To find the suitable NMDA concentration that could stimulate NMDAR moderately but not cause cell damage, we observed the cell viability and cleaved caspase-3 expression in cultured hippocampal neurons incubated with various NMDA concentrations with or without 1.6 MPa $\text{N}_2\text{-O}_2$ exposure. We found that neither $10 \mu\text{M}$ nor $20 \mu\text{M}$ NMDA incubation caused a decrease in cell viability (0.87 (SD 0.14) in $10 \mu\text{M}$, and 0.83 (0.10) in $20 \mu\text{M}$) and an up-regulation in cleaved caspase-3 both in control- and in the hyperbaric group, which suggested that the $10 \mu\text{M}$ or $20 \mu\text{M}$ NMDA was suitable to stimulate NMDA (Figure 3A–C). In addition, hyperbaric $\text{N}_2\text{-O}_2$ exposure significantly prevented the up-regulation of cleaved-caspase-3 induced by $50 \mu\text{M}$ NMDA incubation (0.71 (SD 0.04) vs. 0.59 (0.06)) (Figure 3B–C, $P = 0.021$ vs. $0 \mu\text{M}$ or $50 \mu\text{M}$ control group)), which indicated that hyperbaric exposure may partly inhibit NMDAR.

Figure 1

Changes in EEG and swim test performance of mice exposed to 1.6 MPa N_2 - O_2 mixture. (A) Representative EEG in control and 1.6 MPa N_2 - O_2 groups. (B) The frequency of EEG in each group, $n = 6$ in control group and $n = 9$ in the hyperbaric group (C) Drowning time in the forced swimming test, control vs. hyperbaric exposure, $n = 15$ in each group. The data were analyzed using independent sample *t*-test



HYPERBARIC N_2 - O_2 MIXTURE EXPOSURE INHIBITED NR2B PHOSPHORYLATION AND REDUCED DOWNSTREAM P-CREB LEVEL AFTER NMDA STIMULATION IN CULTURED HIPPOCAMPAL NEURONS

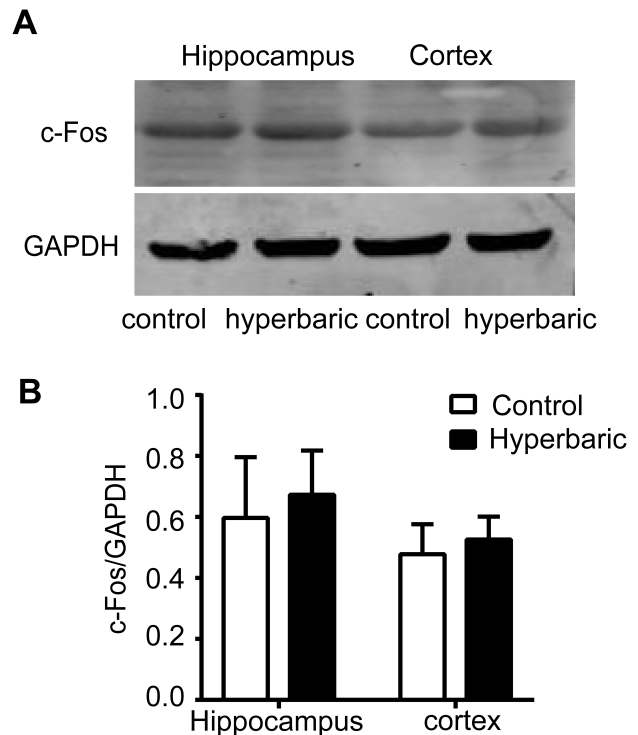
To explore the potential mechanism of changes in the EEG and behavior performance during 1.6 MPa N_2 - O_2 exposure, we determined the expression and phosphorylation levels of GluA1 and NR2B, the subunits of AMPAR and NMDAR respectively. No significant change was seen in the GluA1 and pGluA1 expression after hyperbaric exposure (Figure 4A–B, $P > 0.05$ vs. control group). However, the increased NR2B phosphorylation levels induced by NMDA incubations were significantly inhibited by 1.6 MPa N_2 - O_2 exposure (Figure 4C–D, $P < 0.001$ vs. control group) (10 μ M group, 0.61 (SD 0.03) vs. 0.43 (0.01) and 50 μ M group, 0.62 vs. 0.41 (0.02)). Additionally, the phosphorylation levels of CREB in the cell nucleus, a downstream target of NR2B were also reduced by 1.6 MPa N_2 - O_2 exposure (Figure 4E).

Discussion

The Meyer-Overton hypothesis suggests that a narcotic gas causes disruption of membrane geometry, leading to dysfunction in cell surface proteins and ion channels.

Figure 2

c-Fos expression in the hippocampus and cortex of mice exposed to 1.6 MPa N_2 - O_2 . (A) Representative immunoblotting image of c-Fos and GAPDH. (B) The gray intensity of c-Fos /GAPDH, $n = 5$ in each group. The data were analyzed using the Mann-Whitney U test

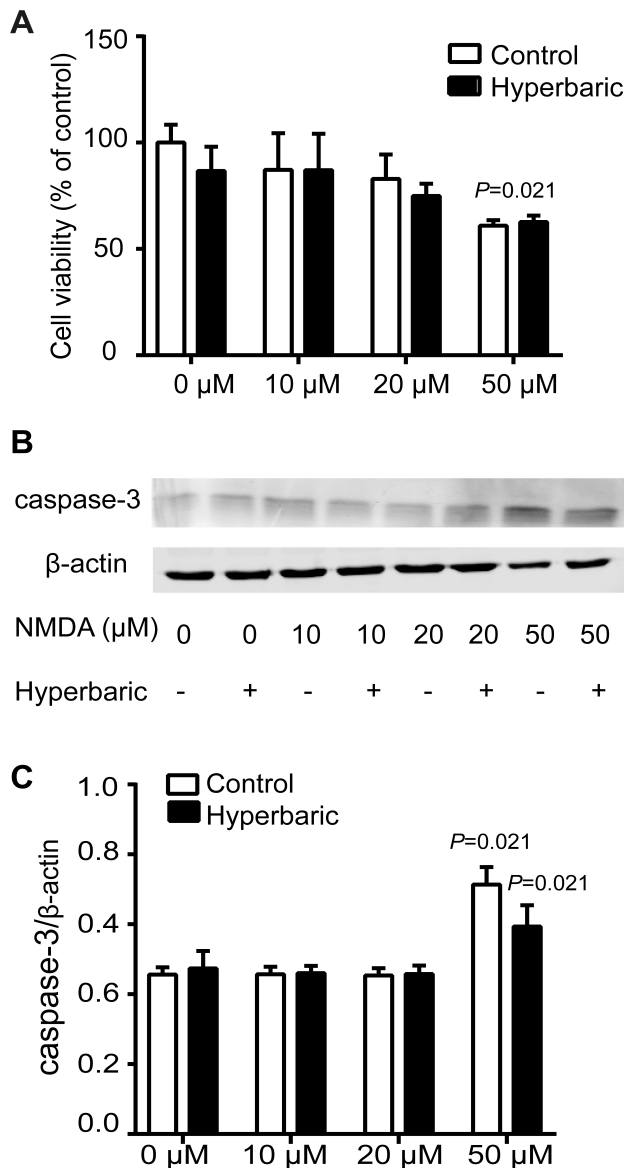


Although this theory has been disproven in favor of other mechanisms such as interactions with neurotransmitter-gated ion channels or other hydrophobic sites on neurons, it still holds that the more lipid soluble an inert gas is, the more narcotic it is.¹⁵ Xenon has been shown to inhibit not only NMDAR but also AMPAR and kainate receptors. We hypothesized that hyperbaric nitrogen will also act on these kinds of proteins.^{3,16} The present study established a mouse model of nitrogen narcosis and investigated the influence of hyperbaric nitrogen on the NMDAR in cultured mouse neurons.

During hyperbaric exposure the EEG showed a reduction in rapid wave activity (alpha and beta waves) and an increase in slow wave activity (theta and delta waves). Furthermore, we found that mice exposed to 1.6 MPa N_2 - O_2 exhibited a drowning time shorter than under normal pressure. These results suggested that exposure to 1.6 MPa N_2 - O_2 could induce CNS inhibition and motor function reduction, two important characteristics of nitrogen narcosis. It is acknowledged that the drowning time result must be interpreted cautiously because increased respired gas density, increased work of breathing, and consequent earlier exhaustion at 1.6 MPa may have contributed to the result independently of a narcotic effect.

Figure 3

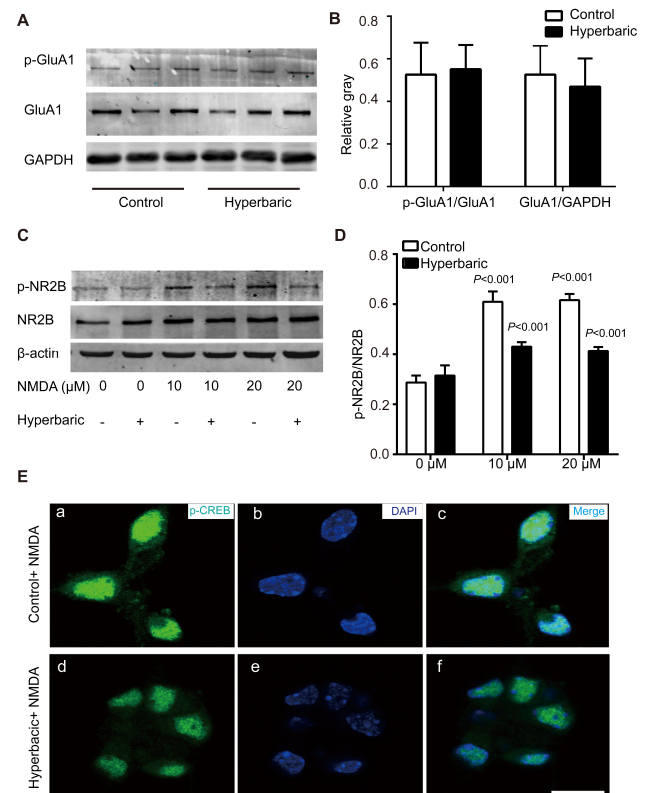
Cell viability and cleaved caspase-3 expression in cultured hippocampal neurons after NMDA incubation with or without 1.6 MPa N_2-O_2 . (A) Cell viability (normalized to 100% of control). (B) Representative immunoblotting of cleaved caspase-3 and β -actin, $n = 4$ in each group. (C) Fold changes in the gray intensity of cleaved caspase-3/ β -actin. The data were analyzed using the Kruskal-Wallis test, and Mann-Whitney U test for multiple comparisons



Humans breathing compressed air at pressures exceeding 0.4 MPa (30 metres' sea water (msw), partial pressure of $N_2 \sim 0.32$ MPa) will experience symptoms resembling those after the use of alcohol, marijuana, and some benzodiazepine drugs, which tend to develop insidiously with depth. Onset of more severe symptoms can render an individual incapable of self-control.¹⁷ In rats, a pressure of 4 MPa of nitrogen was necessary to produce anaesthesia indicated by 100% loss of the righting reflex, and deep nitrogen narcosis was obtained at 75% of the anaesthetic pressure threshold (3 MPa).¹⁸ More subtle cognitive and motor changes have

Figure 4

The increases in the phosphorylated levels of NR2B and CREB in cultured hippocampal neurons after NMDA incubation were inhibited by 1.6 MPa N_2-O_2 exposure. (A) Representative expression of p-GluA1 measured using western blot analysis. (B) Fold changes in the gray intensity of p-GluA1/GluA1 and GluA1/GAPDH, $n = 3$ in each group, the data were analyzed using the Mann-Whitney U test. (C) Representative expression of p-NR2B measured using western blot analysis. (D) Fold changes in the gray intensity of p-NR2B/NR2B, $n = 3$ in each group, one-way analysis of variance and LSD for post hoc test were used. (E) Immunofluorescent images showing the pCREB expression (green in a and d) in the nucleus (blue, DAPI staining b and e), scale bar 25 μ m, $n = 4$ in each group



been reported in humans at 0.3 MPa and 0.8–1.0 MPa in laboratory animals.^{11,19} The present findings on the effect of nitrogen on the EEG and (possibly) motor function in mice exposed to 1.6 MPa N_2-O_2 were consistent with these reports.

A number of experimentally supported mechanisms of inert gas narcosis have been reported at the CNS level and to involve both pre- and post-synaptic effects, many of which are potentially shared by nitrogen and other anaesthetics.^{20,21} NMDAR, the major mediators of glutamatergic neurotransmission, have been recognized as an important target in the induction of anaesthesia. Previous research has shown that 3 MPa nitrogen exposure significantly prevented the increase of extracellular glutamine levels by NMDAR stimulation.²² Additionally, repeated nitrogen exposure disrupted NMDAR function.¹² Consistent with these reports, 1.6 MPa N_2-O_2 exposure in

the present study reduced the phosphorylation levels of NR2B and CREB in cultured hippocampal neurons, which may prevent the lateral redistribution and internalization of NMDA receptors.²² Thus, besides directly affecting the fluidity and structure of the cell membrane, 1.6 MPa N₂-O₂ exposure might inhibit the trafficking as well as the surface distribution of NMDAR. This may thus become a critical mechanism for inhibiting excitatory synaptic function and plasticity.²³

In addition, the present study suggests that hyperbaric nitrogen may act on both synaptic and extra-synaptic NR2B receptors without selectivity because we also found that hyperbaric N₂-O₂ exposure significantly prevented the up-regulation of cleaved-caspase-3 induced by 50 µM NMDA incubation. In previous studies, nitrogen was described to have a pro-GABA activity, to decrease glutamate release, and to have a very poor anti-NMDA activity.^{1,2,11} Vallée et al. found that NMDA receptors remained functional under nitrogen narcosis, as NMDA exposure significantly reversed the decrease of dopamine release induced by nitrogen narcosis.²² In contrast Lavoute et al. reported that the administration of 0.5 nM NMDA produced a significant increase of dopamine at atmospheric pressure, but not during 3 MPa nitrogen exposure; suggesting that the NMDA receptor was inhibited (but perhaps incompletely) during nitrogen narcosis.¹² In the present study, 50 µM NMDA administration caused a decrease in cell viability or up-regulation in caspase-3 expression both in normobaric and hyperbaric conditions, suggesting that the NMDA receptor remained functional, but weak inhibition may exist.

Unfortunately, a number of responses to hyperbaric exposure in neurons including dynamic ion influx and bioelectric activity could not be observed due to technical limitation. An alternative way to detect changes in the expression and phosphorylation of downstream signals for many membrane receptors or channels could help us evaluate their functions. NR2B-containing NMDAR are highly permeable to calcium ions that can trigger numerous intracellular signaling pathways including Ca²⁺/calmodulin-dependent protein kinase II/IV (CaMKII/IV) and CREB, which then will prominently affect neuronal activity.^{24,25} Actually, activation of CaMKII by Ca²⁺ influx through NMDAR could potentiate synaptic efficacy by inducing synaptic insertion and increased single-channel conductance of AMPAR, NR2B anchoring and synapse density.^{26–28} A recent report revealed that CREB-brain derived neurotrophic factor (BDNF) signaling exerted both rapid and slower homeostatic regulation of AMPAR expression.²⁹ Therefore, the present results may indicate that hyperbaric nitrogen exposure is likely to affect the phosphorylation of NR2B and its downstream signaling pathway, and consequently inhibit excitatory transmission in the CNS.

The present study suggests that inhibition of NR2B-containing NMDA receptors during hyperbaric nitrogen exposure may contribute to nitrogen narcosis and resultant

CNS inhibition. However, there are some limitations in this study. More evidence is needed to prove that the hippocampus is the sensitive region. Secondly, whether a similar effect can be observed in cortical neurons or other regions is not known. Moreover, it is unknown if pretreatment with a NR2B blocker can influence the EEG and forced swimming tests, the threshold pressure of nitrogen narcosis in mice, or even the actual NMDA-mediated ion channel slow current of a cultured neuron during hyperbaric exposure.

Conclusion

The present findings indicated that NR2B-containing NMDA receptors were inhibited during nitrogen narcosis.

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