Nitrogen at Raised Pressure Interacts with the GABA<sub>A</sub> Receptor to Produce Its Narcotic Pharmacological Effect in the Rat

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Background: Strong evidence supports the concept that conventional anesthetics, including inhalational agents and inert gases, such as xenon and nitrous oxide, interact directly with ion channel neurotransmitter receptors. However, there is no evidence that nitrogen, which only exhibits narcotic potency at increased pressure, may act by a similar mechanism.

Methods: We compared the inhibitory and sedative effects of γ-aminobutyric acid (GABA) and nitrogen pressure on locomotor activity and striatal dopamine release in freely moving rats and investigated the pharmacologic properties of the GABA-induced and nitrogen pressure–induced narcotic action using the highly selective competitive GABA<sub>A</sub> receptor antagonist bicuculline.

Results: Intracerebroventricular GABA infusion up to 60 µmol or exposure to nitrogen pressure up to 3 MPa decreased to a similar extent striatal dopamine release (r² = 0.899, df = 4, P < 0.01) and locomotor activity (r² = 0.996, df = 28, P < 0.001). However, both agents only showed small effects on striatal dopamine release, reducing dopamine currents by only 12–13% at sedative concentrations. Pretreatment with bicuculline at 0.5, 1, and 2.5 pmol reduced the sedative action of GABA on locomotor activity by 10, 20, and 41%, respectively. Bicuculline in the nanomole range at 1, 2.5, and 5 nmol but not in the picomole range reduced the sedative action of nitrogen pressure by 5, 37, and 73%, respectively. Schild plot analysis is consistent with the fact that bicuculline is a competitive antagonist of both GABA and nitrogen at pressure.

Conclusions: These results suggest that the presynaptic effects of both GABA and nitrogen pressure on striatal dopamine transmission are modest and not mainly involved in their sedative action and that nitrogen at increased pressure may interact directly with the GABA receptor. However, because the antagonistic effect of bicuculline on nitrogen sedation only occurred at much higher bicuculline concentrations than seen with GABA, it is suggested that nitrogen does not compete for the same site as GABA.

MORE than a century after the Frenchman Paul Bert described the narcotic effects of nitrogen at increased pressure, the molecular mechanisms by which the diluent of oxygen in the air produces general narcosis at increased pressure in humans and experimental animals still remain unknown. Because inert gases at pressure produce narcosis in accordance with the Meyer-Overton rule of a high correlation between narcotic potency and lipid solubility, the traditional view has been that nitrogen at increased pressure dissolves in the lipid bilayer of the cellular membrane, occupying or expanding its volume. Since then, lipid theories have been refined and now postulate that lipid occupation or expansion would disrupt indirectly the functioning of neurotransmitter receptors and thereby would disrupt synaptic transmission.

Alternatively, there is growing evidence that the conventional inhalational anesthetic agents that exhibit narcotic potency at normal pressure, including the inert gases xenon and nitrous oxide, interact directly with ion channel receptors, such as the N-methyl-D-aspartate glutamate receptor, the nicotinic acetylcholine receptor, or the γ-aminobutyric acid A (GABA<sub>A</sub>) receptor. However, these agents exhibit narcotic potency at normal pressure, while nitrogen could interact with GABA transmission to produce narcosis at increased pressure. This study directly addresses the question of whether nitrogen could interact with GABA transmission to produce narcosis at increased pressure. We tested this possibility by investigating the inhibitory effects of GABA and nitrogen at increased pressure on locomotor activity and striatal dopamine release, a system of neurotransmission that is well-known to be involved in the control of locomotor activity, and by characterizing the pharmacologic properties of the GABA-induced and nitrogen pressure–induced narcotic action with use of the highly selective competitive GABA<sub>A</sub> receptor antagonist bicuculline.
Electrochemical measurements of dopamine release were made in freely moving rats by differential pulse voltammetry, using a PRG5 polarograph (Taccussel Radiometer, Lyon, France) and a three-electrode potentiostatic system with reference, auxiliary, and carbon multifiber working electrodes. During voltametric recordings, the animals were connected to the polarograph through a flexible cable and a swivel connector. The polarograph was set as follows: scan rate, 10 mV/s; voltage range, 0–500 mV; pulse record, 0.2 s; pulse modulation amplitude, 50 mV; pulse modulation duration, 48 ms.

Carbon multifiber electrodes were made from a rigid rod of 10,000 carbon fibers (AGT4F 10000; Carbone Lorraine, Gennevilliers, France) sharpened at one extremity to reduce the external diameter of the electrode from 1 mm to 50 µm at the tip. The carbon multifiber electrode was encased in an insulating resin, and the tip was exposed using an abrasive disc to shape the active surface of the carbon electrode. Before use, the carbon multifiber electrodes were electrochemically pretreated by applying a triangular wave potential (0–3 V, 70 Hz, 20 s; 0–2 V, 70 Hz, 20 s; and 0–1 V, 70 Hz, 15 s) to increase both their selectivity and sensitivity to dopamine. Before being implanted, the carbon electrodes were calibrated in various solutions of 3,4-dihydroxyphenylacetic acid (DOPAC), ascorbic acid (AA), uric acid (UA), and homovanillic acid (HVA) of 10⁻⁶ to 10⁻³ M. Data acquisition and analysis were made by using an A/D converter interfaced with a computer. Signals were amplified (×10) and recorded every 6 min; dopamine release was quantified by measuring the amplitude of the oxidation currents and expressed as percentage changes. In vivo recording showed oxidation peaks similar to those recorded in dopamine solutions during calibration of the carbon electrodes (peak range, 150–180 mV).

Measurement of Sedation

The sedative effects of GABA or nitrogen pressure were evaluated by using locomotor activity as an index. Locomotor activity was detected using a 12-cm-diameter piezo-electrical sensor (Quartz et Silice, Montreuil, France) that was fixed under the floor of each Perspex activity cage. Data acquisition was made as detailed previously by using a A/D converter interfaced with a personal computer. Signals from the piezo-electrical sensors were sampled at a frequency of 120 Hz on 4-s epochs, were amplified, and were analyzed by performing a fast-Fourier transform. Total activity counts were recorded every minute and were expressed in arbitrary units.

Induction of Sedation by Either Nitrogen or GABA and Pharmacologic Treatments

The freely moving rats were placed in individual Perspex activity cages in a pressure chamber fitted with three viewing windows. Ten minutes after saline or drug pretreatment with the selective GABAA receptor antagonist bicuculline, the animals were compressed with nitrogen of medical grade (Air Products, Paris, France) up to a pressure of 3 MPa at a linear rate of 0.1 MPa/min. Oxygen of medical grade (Air Products) was maintained at a constant partial pressure of 0.025 MPa inside the pressure chamber; a powerful fan ensured mixing of the gases. Carbon dioxide was maintained at less than 300 parts per million by continuously circulating chamber gases through a soda lime canister. To avoid temperature–anesthesia interactions, the temperature inside the pressure chamber was adjusted to maintain rectal temperature at 37 ± 1°C in one additional restrained animal.

To mimic exposure to nitrogen pressure, induction of sedation by GABA was performed by continuous intracerebroventricular infusion. Ten minutes after saline or drug pretreatment with bicuculline, the animals were injected intracerebroventricularly with 60 µmol GABA in a volume of 6 µl saline solution at a rate of 0.2 µl/min using a perfusion apparatus (PHD2000; Harvard Apparatus, Holliston, MA). These experiments were made with...
the pressure chamber maintained at normal pressure; oxygen and carbon dioxide were maintained as described.

GABA and (−)-bicuculline methiodide were purchased from Tocris (distributed by Fisher Bioblock, Illkirch, France). Pretreatment with bicuculline before induction of sedation by nitrogen pressure or GABA infusion was delivered intracerebroventricularly in a volume of 4 μl saline solution at a rate of 1 μl/min using a perfusion apparatus.

Data Analysis
Data from the control records and the GABA and nitrogen pressure experiments were expressed as mean ± SEM. Changes in striatal dopamine release and locomotor activity were analyzed using the t test. Data from the GABA and nitrogen pressure experiments were compared using the Pearson correlation. The inhibitory effects produced by GABA and nitrogen pressure on dopamine release and locomotor activity were fitted by the logistic equation using nonlinear least squares regressions to allow determination of the half-maximal effective GABA concentrations (EC50) and nitrogen pressures (EP50). The dose-dependent effects of the GABA receptor antagonist bicuculline on GABA EC50 and nitrogen EP50 were analyzed using the Schildd plot procedure. Sigmoidal fits with the logistic equation were performed using the Origin® software (Microcal Software Inc., Northampton, MA).

Results
Inhibitory Properties of GABA Infusion and Nitrogen at Pressure
Figure 1 illustrates the inhibitory effects of GABA (n = 5) and nitrogen pressure (n = 5) on striatal dopamine release, compared with control records (n = 5). GABA and nitrogen both showed small effects, reducing striatal dopamine currents to a similar extent by only 13 ± 2% (t test = 8.366, df = 58, P < 0.001) and 12 ± 2% (t test = 7.245, df = 58, P < 0.001), respectively. Statistical analysis showed a significant correlation between the mean values of the inhibitory effects of GABA and nitrogen pressure on striatal dopamine release (r² = 0.899; df = 4, P < 0.01). The fit of the pooled data by the logistic equation yielded a GABA EC50 of 21.04 μmol and a nitrogen EP50 of 1.04 MPa. For the individual experiments, the average GABA EC50 was 17.82 ± 2.91 μmol, and the average nitrogen EP50 was 1.04 ± 0.16 MPa.

As shown in figure 2, GABA (n = 3) and nitrogen at increased pressure (n = 4) both reduced locomotor activity to the same extent, compared with control records (n = 4). GABA and nitrogen reduced locomotor activity by 78 ± 6% (t test = 11.578, df = 208, P < 0.001) and 77 ± 9% (t test = 10.272, df = 238, P < 0.001), respectively. Statistical analysis showed a significant correlation between the mean values of the sedative effects of GABA and nitrogen at increased pressure on locomotor activity (r² = 0.996; df = 28, P < 0.001). The fit of the pooled data by the logistic equation yielded a GABA EC50 of 26.77 μmol and a nitrogen EP50 of 1.52 MPa. For the individual experiments, the average GABA EC50 was 28.85 ± 2.94 μmol, and the average nitrogen EP50 was 1.52 ± 0.26 MPa.

GABA Pharmacology of the GABA- and Nitrogen Pressure–induced Sedative Effects
The GABA pharmacology of the GABA- and nitrogen pressure–induced sedative action was investigated using bicuculline, a highly selective competitive GABA receptor antagonist that can induce arousal and hyperexcitability, such as tonic–clonic seizures or convulsions. The effects of bicuculline infusion in the picomole range and the nanomole range on basal activity are shown in figures 3 and 4, respectively. Administration of bicuculline

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Fig. 1. Inhibitory effects of γ-aminobutyric acid (GABA) and nitrogen pressure on striatal dopamine release. (A) Percentage change in striatal dopamine release versus time expressed in minutes. Compared with control (open squares, n = 5), GABA (closed squares, n = 5) and nitrogen pressure (triangles, n = 5) reduced striatal dopamine release to a similar extent by 12–13% (P < 0.001). Data are expressed as mean ± SEM. The arrow indicates the time at which GABA or nitrogen was applied. Dopamine signals were recorded every 6 min. Statistical analysis showed a significant correlation between the mean inhibitory effects of GABA and nitrogen pressure (r² = 0.899, P < 0.01). (B, C) The fit of the pooled data for the GABA dose–response curve and the nitrogen dose–response curve by the logistic equation yielded a half-maximal effective GABA concentration of 21.04 μmol (B) and a half-maximal effective nitrogen pressure of 1.04 MPa (C).
in the picomole range (fig. 3A) showed no evidence of arousal, hyperexcitability, or locomotor activation ($t$ test = 0.387, $df = 238$, not significant); in the nanomole range, infusion of bicuculline at the higher dose of 5 nmol resulted in convulsions in 20% of the animals (1 in 5), which could have affected the current investigations. To avoid artefactual records and interpretation, the animals that had convulsions were not taken into account. In the 80% of animals that were included in the study, administration of bicuculline resulted in a moderate (18%), nonsignificant increase in locomotor activity ($t^2 = 0.996, P < 0.001$). Data are expressed as mean ± SEM. The arrow indicates the time at which GABA or nitrogen was applied. Locomotor activity was recorded every minute; for clarity of presentation, data were pooled every 6 min. Statistical analysis showed a significant correlation between the mean values of the sedative action of GABA and nitrogen ($r^2 = 0.996, P < 0.001$). (B, C) The fit of the pooled data for the GABA dose–response curve and the nitrogen dose–response curve by the logistic equation yielded a half-maximal effective GABA concentration of 26.77 µmol (B) and a half-maximal effective nitrogen pressure of 1.52 MPa (C).

Fig. 2. Sedative effects of γ-amino butyric acid (GABA) and nitrogen pressure on locomotor activity. (A) Locomotor activity expressed in arbitrary units (A.U.) versus time expressed in minutes. Compared with control (open squares, $n = 4$), GABA (closed squares, $n = 3$) and nitrogen pressure (triangles, $n = 4$) reduced locomotor activity to a similar extent by 77–78% ($P < 0.001$). Data are expressed as mean ± SEM. The arrow indicates the time at which GABA or nitrogen was applied. Locomotor activity was recorded every minute; for clarity of presentation, data were pooled every 6 min. Statistical analysis showed a significant correlation between the mean values of the sedative action of GABA and nitrogen ($r^2 = 0.996, P < 0.001$). (B, C) The fit of the pooled data for the GABA dose–response curve and the nitrogen dose–response curve by the logistic equation yielded a half-maximal effective GABA concentration of 26.77 µmol (B) and a half-maximal effective nitrogen pressure of 1.52 MPa (C).

Fig. 3. Bicuculline antagonized the sedative action of γ-amino butyric acid (GABA). (A) Total locomotor activity expressed in arbitrary units (A.U.; mean ± SEM) during the 30-min period of recording. Pretreatment with bicuculline at 0.5, 1, or 2.5 pmol ($n = 3$ per dose), 10 min before GABA was applied, inhibited GABA sedation ($n = 3$) in a dose-dependent manner. $++P < 0.01, +++P < 0.001$ versus control records; * $P < 0.001$ versus GABA administered alone. Note that bicuculline at 2.5 pmol had no effect on basal locomotor activity. (B) The GABA sedation dose–response curve is shifted to the right by bicuculline at 0.5, 1, and 2.5 pmol, leading to an increase of half-maximal effective GABA concentration ($EC_{50}$) from 26.77 to 51.51 ($B1$), 37.99 ($B2$), and 54.14 µmol ($B3$), respectively. (C) Schild plot analysis yields a linear regression of high reliability ($r^2 = 0.995$) with a slope of 1.085 and a bicuculline $pA_2$ value of 11.62 that corresponds to a $K_i$ value of 2.40 pmol.
Fig. 4. Bicuculline antagonized the nitrogen pressure-induced sedative action. (A) Total locomotor activity expressed in arbitrary units (mean ± SEM) during the 30-min period of recording. Pretreatment with bicuculline at 1, 2.5, or 5 nmol (n = 4 per dose), 10 min before nitrogen was applied, inhibited nitrogen sedation (n = 4) in a dose-dependent manner. *P < 0.05, **P < 0.001 versus control records; **P < 0.001 versus nitrogen pressure when applied alone. Note that bicuculline at 5 nmol had no effect on basal locomotor activity. (B) The nitrogen sedation dose–response curve is shifted to the right by bicuculline at 1, 2.5, and 5 nmol, leading to an increase of half-maximal effective nitrogen pressure (EP50) from 1.52 to 1.66 (B1), 1.93 (B2), and 2.46 MPa (B3), respectively. (C) Schild plot analysis yields a linear regression of high reliability (r2 = 0.994) with a slope of 1.183 and a bicuculline pA2 value of 8.12 that corresponds to a Ki value of 7.59 nmol.

(2) that bicuculline at a dose just sufficient to produce clonic convulsions in 20% of rats resulted in no significant change in locomotor activity; and (3) that intracerebroventricular infusion of bicuculline at 50 nmol (i.e., a dose 10-fold higher than the maximal dose used in the current study) is required to induce hyperactivity and convulsions in the mouse (note the brain weight/dose ratio would be higher in the rat, so that similar effects would be expected to occur at a higher concentration of bicuculline).

The effects of the highly selective GABA_A antagonist bicuculline on the sedative action of GABA on locomotor activity are shown in figure 3. Pretreatment with bicuculline at 0.5, 1, and 2.5 pmol (n = 3 per dose) inhibited GABA sedation (n = 3) in a dose-dependent manner by 10, 20, and 41%, respectively (fig. 3A; −10.978 < t test < −5.189, df = 178, P < 0.001). The fit of the pooled data by the logistic equation shows that the GABA dose–response curve shifted to the right in a parallel manner (fig. 3B), leading to an increase of the GABA EC50 of 26.77 to 31.51, 37.99, and 54.14 μmol, respectively. For the individual experiments, the average GABA EC50s were 28.85 ± 2.94 (GABA alone), 32.43 ± 2.65 (GABA + 0.5 pmol bicuculline), 37.62 ± 4.45 (GABA + 1 pmol bicuculline), and 52.04 ± 2.82 μmol (GABA + 2.5 pmol bicuculline). Schild plot analysis of the series EC50′/EC50, EC50′′/EC50 and EC50′′′/EC50 [in the form log10(EC50′/EC50 − 1), log10(EC50′′/EC50 − 1), log10(EC50′′′/EC50 − 1)] on bicuculline concentration (in the form log10) yields a linear regression of a high reliability (r2 = 0.995), with a slope of −1.085 and a bicuculline pA2 value of 11.62 that corresponds to a Ki value (antilog of −pA2) of 2.40 pmol (fig. 3C).

Pretreatment with bicuculline in the nanomole range but not in the picomole range also reduced the sedative effect of nitrogen at pressure on locomotor activity in a dose-dependent manner, as shown in figure 4. Pretreatment with bicuculline at 1, 2.5, and 5 nmol (n = 4 per dose) inhibited nitrogen sedation (n = 4) in a dose-dependent manner by 5, 37, and 70%, respectively (fig. 4A; −5.837 < t test < −3.204, df = 238, 0.001 < P < 0.01). The fit of the pooled data by the logistic equation shows that the nitrogen dose–response curve shifted to the right in a parallel manner (fig. 4B), leading to an increase of the nitrogen EP50 of 1.52 MPa to 1.66, 1.93, and 2.46 MPa, respectively. For the individual experiments, the average nitrogen EP50s were 1.52 ± 0.26 (nitrogen alone), 1.69 ± 0.17 (nitrogen + 1 nmol bicuculline), 1.94 ± 0.04 (nitrogen + 2.5 nmol bicuculline), and 2.56 ± 0.33 MPa (nitrogen + 5 nmol bicuculline). Schild plot analysis of the series EP50′/EP50, EP50′′/EP50 and EP50′′′/EP50 on bicuculline concentration yields a linear regression of high reliability (r2 = 0.994), with a slope of −1.183 and a pA2 value for bicuculline of 8.12 that corresponds to a Ki value of 7.59 nmol (fig. 4C).
Discussion

Our results show that both GABA infusion and exposure to nitrogen pressure led to a decrease in striatal dopamine release and locomotor activity. The similarities between the inhibitory effects of GABA and nitrogen pressure on dopamine release and locomotor activity suggest that nitrogen at pressure could act, at least partly, through GABA transmission to produce its inhibitory effects. However, while GABA and nitrogen at pressure progressively decreased locomotor activity, they only showed small effects on striatal dopamine release that decreased frankly and then remained at a steady state before the animals evidenced maximal sedation. This led to different nitrogen EP50s for locomotor activity and dopamine release that indicate, in accordance with the current opinion that presynaptic membranes would be less sensitive to general anesthetics than would postsynaptic membranes, that the nitrogen pressure-induced decrease in striatal dopamine release would be poorly involved in the sedative action of nitrogen at increased pressure.

Pretreatment with the highly selective GABA_A receptor antagonist bicuculline reduced the sedative action of both GABA and nitrogen at increased pressure in a dose-dependent manner. Interestingly, Schid plot analysis showed that bicuculline increased GABA EC50 and nitrogen pressure EP50 and shifted the GABA and nitrogen sedation dose-response curves to the right in a parallel manner with a slope of linear regression of 1.085 for GABA and 1.183 for nitrogen pressure. Although Schid plot analysis for competitive antagonism is based on the theoretical assumption that the concentration–response curves for the agonist in the presence of the antagonist must be parallel and the slope of the linear regression for the antagonist must be 1, this is not what happens in reality (because the most competitive antagonist does not behave ideally), and the slope of the linear regression is considered to be optimal within the limits of 0.8–1.2. Our results confirm, with no surprise, that bicuculline acts as a competitive antagonist of GABA and further suggest that it could also competitively antagonize the sedative action of nitrogen at increased pressure.

Because of the Schid plot analysis results and the fact that bicuculline did not significantly affect the baseline of locomotor activity, it is tempting to suggest that nitrogen at pressure could act at the GABA_A receptor for producing its narcotic action. However, the fact that the antagonistic effect of bicuculline on the sedative action of nitrogen at pressure only occurs at much higher bicuculline concentrations than those seen with GABA raises some important questions about whether this reflects a pharmacologic effect, or a physiologic effect that remains extremely difficult to dismiss definitively. It is possible that this effect is indirect and that bicuculline antagonized the sedative action of nitrogen by increasing the general excitability in the central nervous system. Alternatively, the much higher concentration of bicuculline needed to antagonize the narcotic action of nitrogen pressure could indicate, in accordance with the consensus that general anesthetics allosterically modulate ion channel receptors, that nitrogen could bind at multiple ion channel receptors and does not compete necessarily for the same site as GABA at the GABA_A receptor to produce sedation.

In conclusion, the current data are consistent with but did not prove the possibility that nitrogen at pressure may induce its narcotic action partly through a GABA_A mechanism. If such, although the discrete site of action of nitrogen at the GABA_A receptor remains to be identified, the demonstration that the sensitivity of ionotropic receptors to a series of general anesthetics may be modulated by specific mutations in the channel receptor domains—with the receptor sensitivity to anesthetics being increased as hydrophobicity increased—is sufficient to explain the Meyer-Overton correlation between narcotic potency and lipid solubility that is the origin of the lipid theories of inert gas action. However, further experiments are needed to clarify the mode of narcotic action of inert gases at pressure.

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