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Nitrous Oxide (Laughing Gas) Facilitates Excitability in Rat Hippocampal Slices through γ-Aminobutyric Acid A Receptor-mediated Disinhibition

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ALTHOUGH nitrous oxide is safe when administered by anesthesiologists, misuse may result in brain damage either *via* asphyxia[1] or *via* direct effects resulting in vacuole formation in cingulate and retrosplenial cortices under normoxic conditions.[2] The direct toxic effects of nitrous oxide raise the possibility that nitrous oxide toxicity results from excitatory effects of the drug. To determine how nitrous oxide affects neuronal excitability, we studied the actions of nitrous oxide on excitatory synaptic transmission and pyramidal neuron excitability in the CA1 region of rat hippocampal slices using a paired pulse stimulation paradigm and a range of stimulus intensities and intervals.

Materials and Methods

Under halothane anesthesia, 30-day-old male Sprague-Dawley rats were decapitated and the hippocampus was dissected into gassed (95% oxygen: 5% carbon dioxide) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 22, and glucose 10 at 4°–6°C. Transverse slices (500 μm thick) were cut with a vibratome (WPI, Sarasota, FL). After recovering for at least 1 h at 30°C, individual slices were submerged in constant flow recording chamber, perfused with ACSF at 2 ml/min, and maintained at 25°C during the course of the experiment. Procedures for animal studies were approved by the Animal Care and Use Committee at Washington University (St. Louis, Missouri).

Extracellular recordings were obtained from the apical dendritic and pyramidal cell layers of CA1 using 5 to 10 MΩ glass electrodes filled with 1M NaCl. Evoked responses were elicited with 0.1 to 0.2 ms constant current pulses through a bipolar electrode placed in the Schaffer collateral pathway. Synaptic and somatic excitability was evaluated by simultaneously recording excitatory postsynaptic potentials (EPSPs) and population spikes (PSs) using paired-pulse stimulation at intervals of 21 or 42 ms. Dendritic EPSPs (dEPSPs) were measured as maximal slopes of the initial phase of the waveform. PS amplitudes were measured from baseline to the negative peak of the waveform. The amplitude of somatic EPSPs (sEPSP) was measured from baseline to the positive peak of the waveform recorded in the pyramidal cell layer. The ratio of second/first responses (second PS/first PS or second EPSP/first EPSP) was calculated and used to measure paired-pulse plasticity. By comparing relationships among PSs, sEPSPs, and dEPSPs, it is possible to use changes in paired pulse plasticity to monitor γ-aminobutyric acid (GABA)-mediated recurrent inhibition.[3,4,5]

To record *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic responses, 6-cyano-7-nitroquinoxaline-2, 3-dione (30 μM) was added to the ACSF to block non-NMDA receptor components of synaptic transmission. ACSF also included low Mg^{2+} (0.1 mM) and high Ca^{2+} (2.5 mM) to facilitate current flow. Nitrous oxide was bubbled into a reservoir containing ACSF. By decreasing the experimental temperature to 25°C, we were able to maintain neuronal integrity under decreased oxygen concentrations (65%) and study nitrous oxide at concentrations up to 30%. ACSF was preequilibrated at 25°C with 65% oxygen/30% nitrogen/5% carbon dioxide during the control period. After stabilization, nitrogen was replaced with nitrous oxide for 20 min. Nitrous oxide concentrations in the ACSF were monitored with a DATEX 254 airway monitor (Datex, Tewksbury, MA). Only a single slice from each hippocampus was used for an experiment. Data are expressed as mean ± standard error. Statistical significance was determined using paired Student *t* tests or Mann–Whitney U tests.

Results

In preliminary experiments, we found that 30% nitrous oxide was the highest concentration we could administer under atmospheric conditions while preserving stable synaptic transmission for at least 90 min (EPSP changes 95 ± 1%, n = 9).

In the CA1 region, paired-pulse stimulation at short interpulse intervals typically evokes a depression of second PSs evoked after a conditioning first stimulation.

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Table 1. Effect of Nitrous Oxide (N_2O) in the CA1 Region of the Rat Hippocampal Slice

	Dendritic EPSP ratio		Somatic EPSP ratio		PS ratio	
	Pre-drug	Post-drug	Pre-drug	Post-drug	Pre-drug	Post-drug
10% N_2O (n = 5)	0.98 ± 0.03	0.96 ± 0.08	0.44 ± 0.07	0.44 ± 0.06	0.63 ± 0.02	0.64 ± 0.01
30% N_2O (n = 6)	0.85 ± 0.07	0.86 ± 0.08	0.53 ± 0.08	0.59 ± 0.05	0.39 ± 0.05	0.62 ± 0.09*
PTX (n = 5)	0.80 ± 0.03	0.81 ± 0.04	0.30 ± 0.05	0.31 ± 0.03	0.53 ± 0.06	0.85 ± 0.07*
30% N_2O/PTX (n = 5)	0.85 ± 0.10	0.85 ± 0.09	0.36 ± 0.03	0.37 ± 0.04	0.91 ± 0.04	0.93 ± 0.06
30% N_2O at 42 mec (n = 6)	0.89 ± 0.08	0.93 ± 0.07	0.85 ± 0.09	0.87 ± 0.09	0.88 ± 0.11	0.87 ± 0.12
30% N_2O/gabazine (n = 5)	0.78 ± 0.06	0.79 ± 0.06	0.43 ± 0.08	0.44 ± 0.03	0.89 ± 0.07	0.88 ± 0.07

Values are mean ± standard error (n refers to number of slices).

* $P < 0.05$ by paired Student *t*-test compared to the pre-drug ratios.

EPSP = excitatory postsynaptic potentials; PS = population spikes; PTX = picrotoxin.

This second PS inhibition reflects recurrent network inhibition and can be used as a monitor of regional neuronal excitability. We found that 30% nitrous oxide had no effect on the first PSs, but significantly facilitated the paired second PS when evoked at an interval of 21 ms (table 1, fig. 1). This enhanced pyramidal neuron excitability did not result from increased excitatory synaptic input to CA1 because the slopes of initial and second dEPSPs were not altered by 30% nitrous oxide. In addition, nitrous oxide had no effect on the ratio of first PS to first dEPSP, an index of the ability of a test synaptic input to drive neuronal firing. Similarly, the ratio of second sEPSPs to first sEPSPs was unchanged, indicating that nitrous oxide did not affect current flow between dendrites and cell body during paired stimulation. The effects of nitrous oxide on paired-pulse plasticity were not observed at a concentration of 10%.

To assess whether changes in γ-aminobutyric acid-mediated inhibition contribute to nitrous oxide-mediated paired-pulse facilitation of PS, picrotoxin (PTX), a $GABA_A$ receptor antagonist, was used. Because 1 μM PTX, dissolved in ethanol, induced epileptiform discharges, making analysis difficult, we used 0.5 μM PTX. Similar to nitrous oxide, PTX enhanced second PSs but did not change second dEPSPs. To assess whether PTX occludes the actions of nitrous oxide, 30% nitrous oxide was applied after pretreatment with PTX (fig. 2). In the presence of PTX, nitrous oxide had no effect on the second PS, and the ratio of second PS/first PS remained equivalent at a 21-ms interstimulus interval. Similarly, the ratio of second dEPSPs/first dEPSPs was unaffected by nitrous oxide in the presence of PTX, as was the ratio of second sEPSPs/first sEPSPs. The ratio of second PS/first PS in the presence of nitrous oxide was significantly less

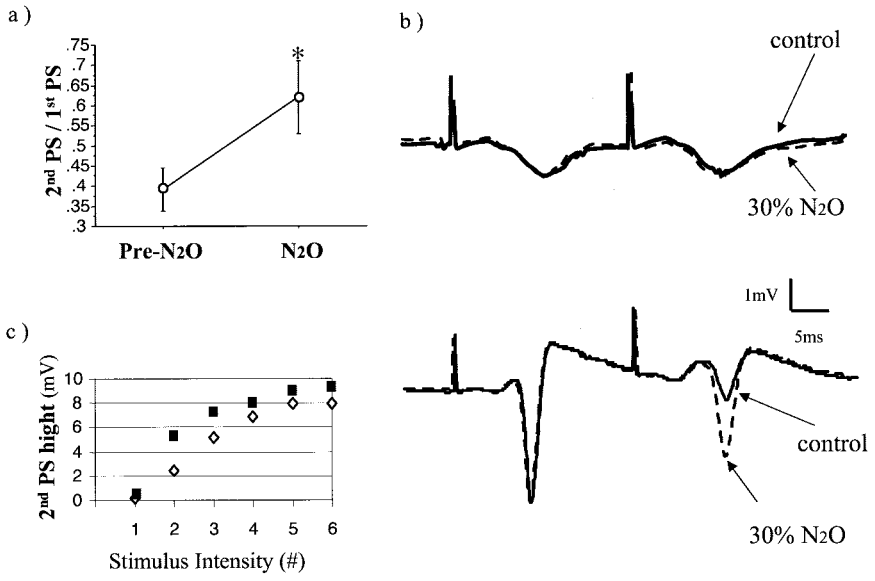

Fig. 1. Effects of 30% nitrous oxide on excitatory synaptic responses. a) Nitrous oxide significantly increased the ratio of second population spike/first population spike ($P < 0.05$ by paired Student *t* test, n = 6). b) Traces show representative excitatory postsynaptic potentials (*upper*) and population spikes (*lower*) depicted before (*solid lines*) and 20 min after (*dotted lines*) nitrous oxide administration. Nitrous oxide facilitated the second population spike with no effect on first population spike amplitude or first and second excitatory postsynaptic potential slope. c) Input-output curves for second population spike amplitude recorded before (*diamonds*) and 20 min after (*squares*) nitrous oxide administration in a single slice. The increased amplitude of the second population spike was greater than the expected value calculated from the control input-output curves at the level of 50% to 60% of the maximal second population spike amplitude.

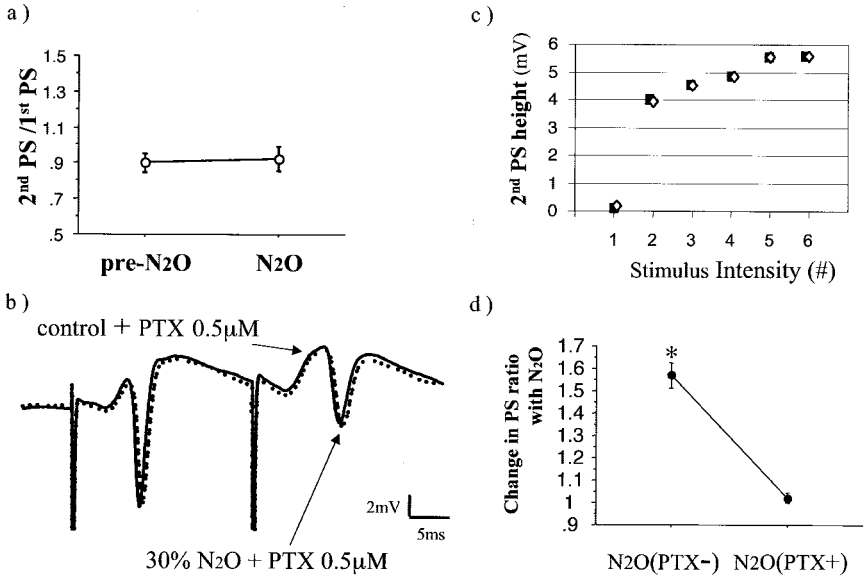

Fig. 2. Effects of picrotoxin and nitrous oxide on population spike amplitude. a) In the presence of 0.5 μM picrotoxin, a γ-aminobutyric acid A receptor antagonist, the ratio of second population spike/first population spike remained unchanged at a 21 ms interstimulus interval (P = NS by paired Student t test, n = 5). b) In the presence of 0.5 μM picrotoxin, traces depict representative population spike before (*solid*) and 20 min after (*dotted*) nitrous oxide administration. Nitrous oxide did not change the second population spike in the presence of picrotoxin. c) Input-output curves for second population spike amplitude recorded before (*diamonds*) and 20 min after (*squares*) nitrous oxide administration in a single slice in the presence of 0.5 μM picrotoxin. Nitrous oxide did not change second population spike based on input/output curves. d) Nitrous oxide (picrotoxin) significantly increased the population spike ratio (ratio of second population spike/first population spike in the presence of nitrous oxide over the ratios of second population spike/first population spike in pre-nitrous oxide) (P < 0.01 by Mann–Whitney U test, n = 5, 6, respectively).

in slices pretreated with PTX compared with slices not treated with PTX ($P < 0.01$ by Mann–Whitney U test). Similar results were obtained with another $GABA_A$ receptor antagonist, gabazine (SR 95531), at a concentration of 0.02 μM.

Because recurrent inhibition in CA1 is time-dependent, we also examined effects of nitrous oxide at longer interpulse intervals. When paired responses were evoked using an interval of 42 ms, 30% nitrous oxide had no effect on the ratio of second PS/first PS or on the ratio of second dEPSPs/first dEPSPs (fig. 3). Similarly, the ratio of second sEPSPs/first sEPSPs was also unaltered. Although clinically relevant concentrations of nitrous oxide inhibit NMDA receptors,[2,6] 30% nitrous oxide had no effect on isolated NMDA receptor-mediated EPSPs (fig. 4). To verify that these EPSPs are mediated by NMDA receptors and to compare the effects of nitrous oxide to another anesthetic that inhibits NMDA receptors, we

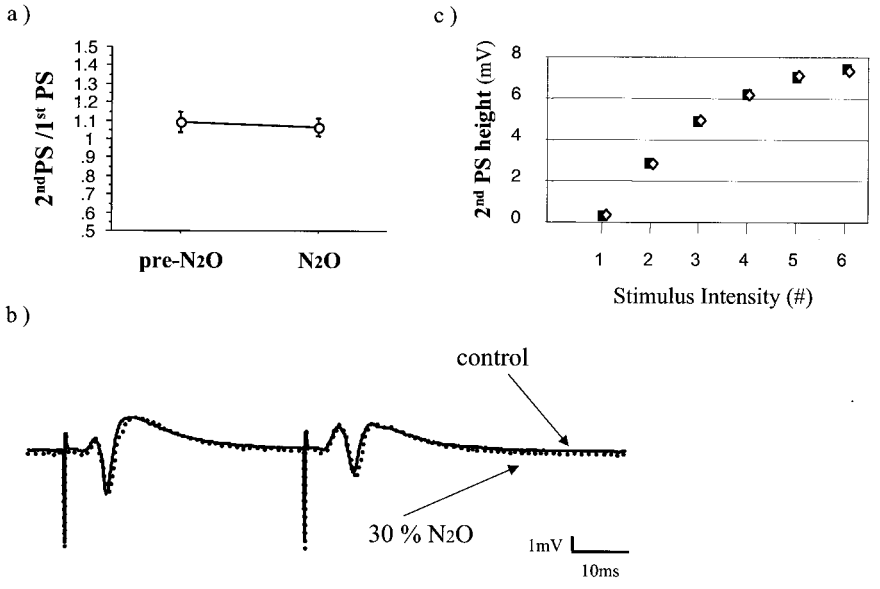

Fig. 3. Effect of 30% nitrous oxide on second population spike at 42-ms interstimulus intervals. a) The ratios of second population spike/first population spike remained stable at a 42 ms interstimulus interval (pre-nitrous oxide *versus* nitrous oxide; 0.88 ± 1.10 *versus*. 0.87 ± 0.12; P = NS by paired Student t test, n = 6). b) At an interstimulus interval of 42 ms, traces show representative population spike before (*solid*) and 20 min after (*dotted*) nitrous oxide administration. c) The graph shows input-output curves for second population spike amplitude recorded before (*diamonds*) and 20 min after (*squares*) nitrous oxide administration from a single slice at an interstimulus interval of 42 ms. Nitrous oxide did not change second population spike based on input/output curves.

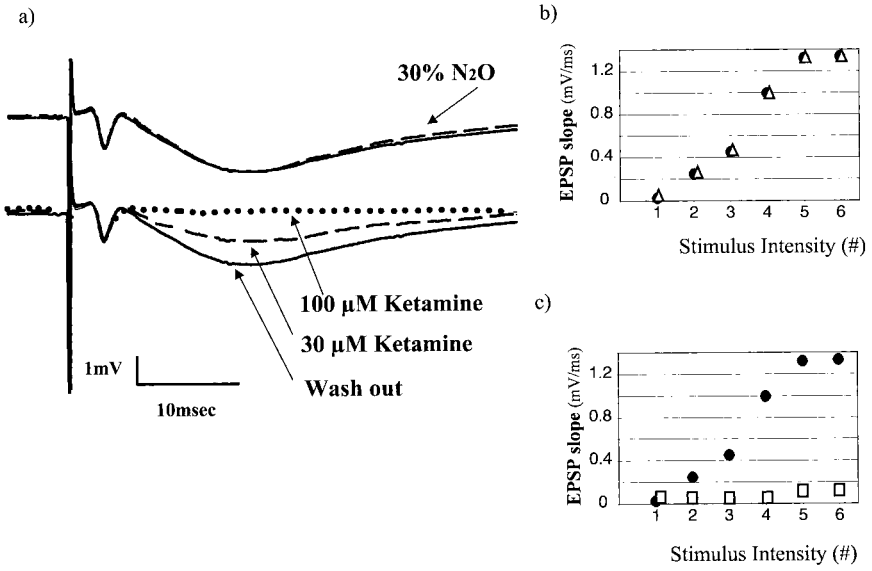

Fig. 4. Differences between nitrous oxide and ketamine on *N*-methyl-D-aspartate receptor-mediated excitatory postsynaptic potentials. Excitatory postsynaptic potentials mediated by *N*-methyl-D-aspartate type glutamate receptors were evoked by single pulses administered to the Schaffer collateral pathway. a) Traces show representative excitatory postsynaptic potentials (*upper*) before (*solid*) and 20 min after (*dotted*) nitrous oxide administration. The *lower* traces show representative excitatory postsynaptic potentials (*lower*) after washing out nitrous oxide (*solid*) and 20 min after (*thick dotted*) ketamine administration. At 100 µM, ketamine completely blocked *N*-methyl-D-aspartate mediated excitatory postsynaptic potentials. b) The graph shows input-output curves for excitatory postsynaptic potential slopes recorded before (*circles*) and 20 min after (*triangles*) nitrous oxide application in a single slice. Nitrous oxide 30% did not change *N*-methyl-D-aspartate mediated excitatory postsynaptic potentials. c) The graph shows input-output curves for excitatory postsynaptic potential slopes recorded before (*circles*) and 20 min after (*squares*) 100 µM ketamine in a single slice.

administered ketamine to the same slices after washing out nitrous oxide. Ketamine inhibited NMDA receptor-mediated EPSPs in a concentration-dependent manner with complete inhibition at 100 µM. Unlike nitrous oxide, however, up to 100 µM ketamine failed to alter basal synaptic responses or paired-pulse responses at an interpulse interval of 21 ms (data not shown, n = 3).

Discussion

In the presence of 30% nitrous oxide, dEPSPs and sEPSPs and first PSs were not altered, whereas second PSs were increased. This suggests that at low concentrations, nitrous oxide does not alter excitatory input to CA1 or electrical conductance of synaptic inputs along dendrites but does enhance somatic excitability after synaptic activation. Because this paired pulse facilitation of PSs was occluded by PTX and gabazine and not observed at a longer intersimulation interval, a decrement in GABA-mediated inhibition at the pyramidal neuron soma appears to contribute to the excitability changes induced by nitrous oxide.

When excitatory synaptic inputs and dendritic conductances are intact, the somatic inhibition is expressed as a decrease in the ratio of second PS/first PS at short interpulse intervals. In naïve slices, the ratio of second PS/first PS at 21 ms is usually small, reflecting the activation of inhibitory inputs during the initial stimulus. Under basal conditions, the reduction in second PS results in part from a decrease in dendritic conductance as reflected by a smaller second sEPSP. However, the paired-pulse depression of second PS persists even after increasing sEPSPs to the same size as the conditioning first sEPSP, indicating the presence of robust recurrent collateral inhibition of the soma diminishing the ability of pyramidal neurons to fire.

The effects of recurrent inhibition can be diminished either by prolonging the interpulse interval or by blocking $GABA_A$ receptor inhibition. At a 42 ms interstimulus interval, PTX had no effect on second PS amplitudes in control slices, suggesting a short duration of $GABA_A$ receptor-mediated inhibition. Similarly, 30% nitrous oxide did not alter second PSs at a 42 ms interstimulus interval. We also observed that 30% nitrous oxide did not change second PSs in the presence of PTX, suggesting that 30% nitrous oxide facilitates somatic excitability through γ-aminobutyric acid-mediated disinhibition.

In our study, nitrous oxide did not alter second sEPSPs in the paired pulse paradigm, suggesting little effect of nitrous oxide on dendritic to soma conductance. Accordingly, it could be speculated that nitrous oxide more selectively affects γ-aminobutyric acid-mediated interneurons whose axons terminate on pyramidal cell bodies.[7,8]

Although use of up to 30% nitrous oxide in the current study only partially characterizes the actions of nitrous oxide, future experiments using higher pressures should

enable the study of effects of nitrous oxide at anesthetic concentrations.

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