ANESTHESIOLOGY

Nitrous Oxide Impairs Axon Regeneration after Nervous System Injury in Male Rats

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ABSTRACT

Background: Nitrous oxide can induce neurotoxicity. The authors hypothesized that exposure to nitrous oxide impairs axonal regeneration and functional recovery after central nervous system injury.

Methods: The consequences of single and serial *in vivo* nitrous oxide exposures on axon regeneration in four experimental male rat models of nervous system injury were measured: *in vitro* axon regeneration in cell culture after *in vivo* nitrous oxide administration, *in vivo* axon regeneration after sharp spinal cord injury, *in vivo* axon regeneration after sharp optic nerve injury, and *in vivo* functional recovery after blunt contusion spinal cord injury.

Results: *In vitro* axon regeneration 48 h after a single *in vivo* 70% N_2O exposure is less than half that in the absence of nitrous oxide (mean ± SD, 478 ± 275 um; n = 48) *versus* 210 ± 152 um (n = 48; $P < 0.0001$). A single exposure to 80% N_2O inhibits the beneficial effects of folic acid on *in vivo* axonal regeneration after sharp spinal cord injury (13.4 ± 7.1% regenerating neurons [n = 12] *vs.* 0.6 ± 0.7% regenerating neurons [n = 4], $P = 0.004$). Serial 80% N_2O administration reverses the benefit of folic acid on *in vivo* retinal ganglion cell axon regeneration after sharp optic nerve injury (1277 ± 180 regenerating retinal ganglion cells [n = 7] *vs.* 895 ± 164 regenerating retinal ganglion cells [n = 7], $P = 0.005$). Serial 80% N_2O exposures reverses the benefit of folic acid on *in vivo* functional recovery after blunt spinal cord contusion (estimate for fixed effects ± standard error of the estimate: folic acid 5.60 ± 0.54 [n = 9] *vs.* folic acid + 80% N_2O 5.19 ± 0.62 [n = 7], $P < 0.0001$).

Conclusions: These data indicate that nitrous oxide can impair the ability of central nervous system neurons to regenerate axons after sharp and blunt trauma.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Nitrous oxide exposure can induce spinal cord neurodegeneration and related neuropathy in humans rendered susceptible by genetic or acquired risk factors
- The effects of nitrous oxide on the regeneration of nervous system following trauma has not been previously reported

What This Article Tells Us That Is New

- In *in vitro* and *in vivo* experimental models of male rats, nitrous oxide exposure impairs folic acid-induced axonal regeneration of dorsal root and retinal ganglion neurons
- The beneficial effects of folic acid on functional recovery following spinal cord contusion in male rats are hindered by co-administration of nitrous oxide
- These experiments suggest that nitrous oxide can interfere with axonal regeneration and functional recovery following central nervous system injury

Methionine is the single carbon donor in mammalian cells and is an essential participant in a diversity of metabolic pathways, including myelin and neurotransmitter synthesis and regulation of DNA transcription. Conversion of serine to glycine provides a methyl group for the synthesis of methionine from homocysteine.[1] The methyl group binds tetrahydrofolate to generate 5,10-methylenetetrahydrofolate, which is reduced to 5-methyltetrahydrofolate by 5,10-methylenetetrahydrofolate reductase. The methyl group is then transferred from 5-methyltetrahydrofolate to cobalamin to produce methylcobalamin, the final methyl group donor for methionine synthesis. Accordingly, methionine synthase requires 5-methyltetrahydrofolate as its single carbon source and is irreversibly inactivated by nitrous oxide with oxidation of its cobalamin cofactor.[1] Parenteral folic acid administered before and after spinal cord injury produces a tenfold or greater dose-dependent improvement in axon regeneration in the adult central nervous system, with peak effects observed at 80 μg/kg folic acid.[2] At higher folic acid doses beneficial effects diminish with no toxicity observed. Eighty percent N_2O in 20% oxygen administered 3 days before spinal cord injury for 4 h, and thereafter every other day for 2 h for 2 weeks, antagonizes the beneficial effects of folic acid on axon regeneration after sharp spinal cord injury.[3] To further resolve the effects of nitrous oxide on axon regeneration after injury to the nervous system,

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we hypothesized that single and serial *in vivo* nitrous oxide exposures impair axon regeneration in four experimental models of nervous system injury: *in vitro* axon regeneration in cell culture after *in vivo* nitrous oxide administration, *in vivo* axon regeneration after sharp spinal cord injury, *in vivo* axon regeneration after sharp optic nerve injury, and *in vivo* functional recovery after blunt contusion spinal cord injury.

Materials and Methods

Institutional Review and Animal Care

With the approval of the University of Wisconsin Animal Care and Use Committee (Madison, Wisconsin), and in compliance with published Public Health Service-National Institutes of Health guidelines, all experiments were conducted with 4- to 8-week-old male Sprague–Dawley rats weighing 200 to 250 g (Harlan Laboratories, Inc., USA) that were housed in approved facilities at the University of Wisconsin-Madison (Madison, Wisconsin) staffed by licensed veterinarians. Female rats were conserved to maintain an animal colony sufficient to perform the experimental protocols. Animals were fed Harlan Rodent Diet #8604 (Harlan Laboratories, Inc.), containing B_6 (95 mcg/kg), B_{12} (51.20 mcg/kg), folic acid (2.72 mg/kg), and methionine (0.42% by weight) with unrestricted access other than on the morning of surgery. All experiments were carried out between 8:00 AM and 5:00 PM by investigators blinded to treatment. The animals used in this study were randomized to experimental conditions. Surgical preparation and assessment were randomized between control and treated animals on each experimental day. The number of animals and animal suffering were reduced maximally in all experiments. The percentage of animals that did not survive nitrous oxide exposure, folic acid administration, and surgery to establish the models, and to apply stains to regenerating axons, was less than 5%. Rats did not experience unexpected lethality in the study, and animals were euthanized according to our institutional animal care and use committee guidelines.

Nitrous Oxide Administration

Nitrous oxide in oxygen was administered by placing wire cages housing two to four rats each into a 114 l chamber within a lighted, quiet, negative pressure chemical fume hood. The sealed chamber lid comprised two one-way ports. The ingress port permitted administration of gases, and the egress port permitted pressure equilibration and scavenging of gases. After placing the cages in the exposure chamber, the chamber was brought to 100% oxygen at 6 l/min delivered from a size 200 high-pressure industrial 43 l cylinder (Material Distribution Service, University of Wisconsin, Madison, Wisconsin) yoked to a SurgiVet, Inc. (USA), model #9901544 mixer, and confirmed by a gas analyzer (Ohio 5100 Oxygen Monitor; Ohmeda, Inc., USA) with its transducer placed within the chamber after calibration to 21% and 100% fraction of inspired oxygen (FIO_2) at the outlet of anesthesia machine. Nitrous oxide was introduced through the ingress aperture to reach the target FIO_2 concentration. The FIO_2 was continuously monitored and held stable for the duration of each experiment by adjustment of oxygen and nitrous oxide flows. A minimal flow rate of 1 l/min was maintained throughout the exposure interval to assure a slight positive pressure to preclude inward leak of ambient air. The humidity of the atmosphere within the fume hood was 54%, and the temperature was 25°C. Rats were allowed free access to water, feed, bedding, and space to move and interact throughout the exposure interval. Once the exposure interval was complete, the chamber was again flushed to 100% oxygen, and cages housing the animals were removed.

In Vitro Axon Regeneration after *In Vivo* Nitrous Oxide Administration

Protocol. In protocol 1a, control bilateral L4 and L5 dorsal root ganglia were harvested from four rats euthanized with a 10 ul intraperitoneal mixture of pentobarbital sodium 390 mg/ml and phenytoin sodium 50mg/ml (Beuthanasia Schering-Plough Animal Health, Inc., USA) without antecedent nitrous oxide exposure or sciatic nerve injury (fig. 1; Supplemental Digital Content fig. 1, 1a, http://links.lww.com/ALN/C21). In protocol 1b, bilateral L4 and L5 dorsal root ganglia were harvested from four rats immediately after a single administration of 70% N_2O in 30% oxygen for 2 h and no sciatic nerve injury (Supplemental Digital Content fig. 1, 1b, http://links.lww.com/ALN/C21). In protocol 1c, bilateral L4 and L5 dorsal root ganglia were harvested from four rats after serial administration of 80% N_2O in 20% oxygen for 2 h on each of 3 days before harvest and no sciatic nerve injury (Supplemental Digital Content fig. 1, 1c, http://links.lww.com/ALN/C21). In protocols 1d and 1e, the left sciatic nerve was transected by a sharp scissor through an incision midway between the knee and hip joints with ketamine and xylazine 10:1 0.2–0.3 ml intraperitoneal anesthesia (ketamine [40 to 80 mg/kg; Clipper Distributing Company, LLC, USA] and xylazine [5 to 10 mg/kg; Bimeda-MTC, Animal Health Inc., USA]). The limb wound was closed with Vicryl 4-0 nylon sutures (Ethicon, Inc., USA), and the rats were observed until full recovery from anesthesia. In protocol 1d, L4 and L5 dorsal root ganglia ipsilateral to the left sciatic nerve injury were harvested from eight rats euthanized on day 3 after injury on day 1 (Supplemental Digital Content fig. 1, 1d, http://links.lww.com/ALN/C21). In protocol 1e, the L4 and L5 dorsal root ganglia ipsilateral to the left sciatic nerve injury were harvested from eight rats exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (Supplemental Digital Content fig. 1, 1e, http://links.lww.com/ALN/C21).

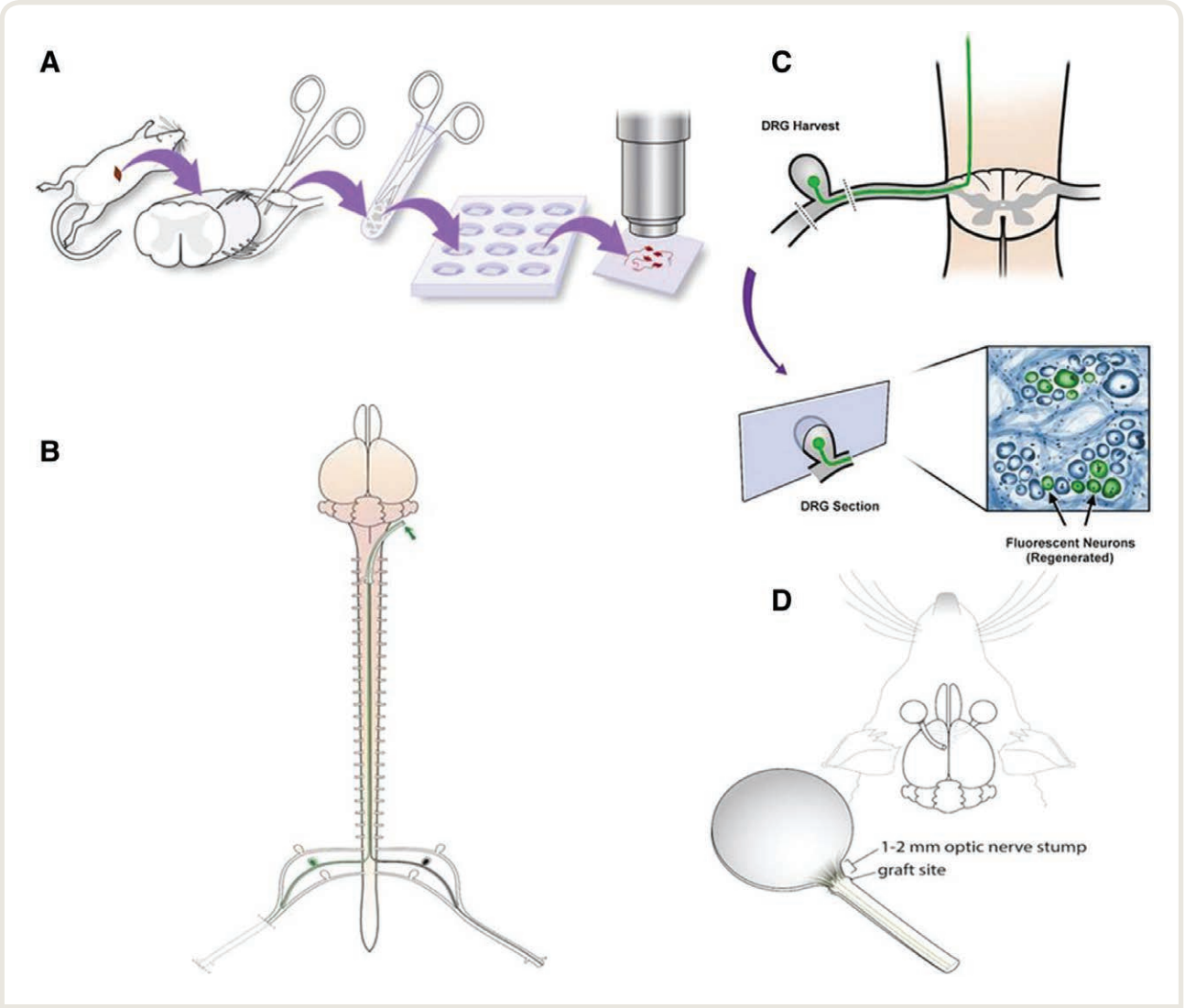

Fig. 1. (*A*) Schematic diagram of *in vitro* axon regeneration in culture after *in vivo* nitrous oxide administration experiments. Axon length and the number of cells with an axon length greater than 300 um were measured under 20× magnification after dorsal root ganglia (DRG) cell harvest, dissociation, culture and immune-staining. (*B*) Schematic diagram of *in vivo* axon regeneration after sharp spinal cord injury experiments. Dorsal root ganglia preparation after bilateral C3 dorsal column lesion and a sciatic nerve graft (*green arrow*). At 2 weeks, fluorescent tracer is applied to the distal graft, and is detected 2 days later in lumbar dorsal root ganglia neuronal axons that have grown into the graft (*green*). The fluorescent tracer is only taken up in regenerated neurons. (*C*) Regenerated fluorescent neurons (*green*) are counted under 20× fluorescent microscope magnification after dorsal root ganglia harvest and sectioning. (*D*) Schematic diagram of *in vivo* axon regeneration after sharp optic nerve injury experiments. Retinal ganglion cells with regenerated axons are counted under 20× fluorescent microscope magnification after retinal extraction and transfer to glass slides.

Dorsal Root Ganglia Cell Harvest, Culture, Immunostaining, and Analysis. All experiments were performed in triplicate, with cells from 16 bilateral L4 and L5 dorsal root ganglia harvested from four rats comprising each of three independent replicate pools for subsequent culture in uninjured protocols 1a, 1b, and 1c, and cells from 16 unilateral L4 and L5 dorsal root ganglia harvested from eight rats comprising each of three independent replicate pools for subsequent culture in injured protocols 1d and 1e.[4] To dissociate the pooled cells, excised ganglia were placed in 4°C media comprising 48.5 ml Roswell Park Memorial Institute media (Hyclone, Thermo Scientific, Inc., USA) and 1.5 ml B27 (GIBCO, Invitrogen, Inc., USA), and mechanically disrupted by a micro-dissecting scissor before washing (fig. 1A). After removal of the media, the dorsal root ganglia fragments were washed twice with Ca^{++} and Mg^{++} free 0.1M phosphate buffered saline (Hyclone). The pooled ganglia from each replicate were added to 2 ml phosphate buffered saline containing 200 μl dispase (Life Technology, USA) and 100 μl collagenase (35mg/ml; Sigma, LLC, USA), and incubated

at 37°C in 5% CO_2 (Thermo Scientific, Inc., Napco series 8000 Ohforma Sterile Cycle CO2 Incubator) for 10 min after gentle mixing with a Pasteur pipette. Twenty-five microliters of DNAse (Sigma, LLC) were added and the ganglia incubated for an additional 10 min at 37°C.

After dissociation, cellular debris and non-neuronal cells were removed by centrifugation (CS-6 centrifuge, Beckman-Coulter, Inc., USA) at 500 rpm for 5 min in 5 ml Roswell Park Memorial Institute media–fetal bovine serum solution comprising 47.5 ml Roswell Park Memorial Institute media and 2.5 ml fetal bovine serum (Hyclone). The supernatant was aspirated and the cells centrifuged a second time at 500 rpm for 5 min after resuspension in 5 ml fresh Roswell Park Memorial Institute media–fetal bovine serum. After the second aspiration, the cells from each replicate were resuspended by pipette in 1.6 ml Roswell Park Memorial Institute-B-27 media. Thus, 100 μl of media contained the dissociated cells of each of 16 dorsal root ganglia to yield the cells of one dorsal root ganglia for each of 16 wells on two 12-well tissue sterilized culture plastic plates free of pyrogens, DNA, DNAse, RNA, and RNAse (Techno Plastic Products, Pvt Ltd., Switzerland); that is, eight wells were used per plate per replicate. Glass coverslips (Thermo Scientific, Inc.) were used as substrates for cell culture. Coverslips were sterilized in nitric acid for 14 h, stored in 100% ethanol, and dried in a sterile hood before placing one coverslip in each well of the 12-well plates (fig. 1A). The coverslips were then immersed in 100 mg/ml poly D-lysine (Sigma, LLC) and placed in the carbon dioxide incubator for 12 h at 37°C. Five hours before plating the cells for culture, the poly D-lysine was aspirated, and the coverslips were washed three times with phosphate buffered saline and immersed in laminin (L2020 100 mg/ml in phosphate buffered saline; Sigma, LLC) for 4 h. After aspiration of the laminin and three phosphate buffered saline washes, 330 ul of Roswell Park Memorial Institute-B27 media was placed in each well, and 100 ul of the 1.6 ml stock suspended cell suspension was added by pipette to each well at room temperature such that each well and coverslip had a paired duplicate for analysis. The plates comprising wells containing coverslips and cells were placed in the incubator at 37°C, and refed once every 24 h with Roswell Park Memorial Institute-B27 media after gentle aspiration of the preceding media.

Duplicate, paired coverslips were removed for fixation, image acquisition and scoring at 5, 10, 13, 17, 24, 36, and 48 h for protocols 1a, 1c, 1d, and 1e, and at 13, 24, and 48 h for protocol 1b. After aspiration of the media, the cells were washed three times with Roswell Park Memorial Institute-27 media, and fixed in 250 ul 4% paraformaldehyde (Sigma, LLC) at 4°C for 30 min. The paraformaldehyde was then removed and the coverslips rinsed twice with phosphate buffered saline with 0.01% Triton X-100 (Acros Organics, Inc. USA). The cells were blocked with a solution comprising 5 ml of 10× phosphate buffered saline, 1 ml 10% Triton X, 2.5 ml 5% fetal bovine serum, and water. Dorsal root ganglia neurons were stained with Sigma T-8660 mouse monoclonal anti-bIII tubulin antibody (Sigma, LLC) diluted to 1:1,000 with blocking solution, by adding 500 ul of the mixture to each well followed by incubation at room temperature for 30 min. After removal of the anti-bIII tubulin antibody and three washes in phosphate buffered saline with 0.01% Triton X-100, Alexa Fluor donkey anti-mouse secondary IgG antibody (Alexa Fluor 594; Invitrogen, Inc., USA) 0.2 mg/ml was diluted 1:1,000 in blocking solution, placed on the cells, and incubated at room temperature for 30 min in the dark, before three further washes in phosphate buffered saline with 0.01% Triton X-100. The coverslips were then removed from the wells, rinsed twice with distilled water, and mounted cell side down on glass slides (Thermo Scientific, Inc.) with 10× polyvinyl alcohol in phosphate buffered glycerol with 0.1% sodium azide (Fluoromount G, Electron Microscopy Sciences, USA) one drop per cover slip, dried for 10 min, and frozen at −80°C (Thermo Scientific Forma 916, USA) thereafter.

Quantitative analyses of the primary outcomes of axon length and the percentage of cells with axons greater than 300 um were performed by identification of the longest axon from each cell for measurement. Images were acquired using Axiovision software (Zeiss, LLC, USA) for an Axioscope 20 fluorescent microscope (Zeiss, LLC) at 20× magnification by two observers blinded to treatment and time interval. Each coverslip was divided into four quadrants, and four images containing at least one complete cell each were selected at random in each quadrant by tracking the longest axon away from each cell body of each image to ensure that the entire length was measurable. When at least one cell was found in an image, its axon was measured. A score of 0 indicates that a cell has been found, but that it has no axon. Multiple cells found within a single image were counted separately. Rarely were more than five cells identified within an image. The percentage of cells greater than 300 um was calculated with a denominator of all cells counted per coverslip/time interval/replicate experiment divided into the number of cells with an axon greater than 300 um on the same coverslip. For all values, the measurements of the two blinded observers were averaged to a single value for each quadrant, and the average axon lengths and percentage cells greater than 300 um were calculated for each of the three replicates. ImageJ Software (http://imagej.nih.gov/ij/; accessed July 27, 2018) was used to calibrate the measurement scale using the scale image taken at 20× magnification, and to archive the photomicrographs.

In Vivo Axon Regeneration after Sharp Spinal Cord Injury

Protocol. In protocol 2a, 14 control rats received no folic acid or nitrous oxide before or after spinal cord surgery and injury (fig. 1B; Supplemental Digital Content fig. 2, 2a, http://links.lww.com/ALN/C22). In protocol 2b, 12 rats received 80 μg/kg intraperitoneal folic acid each day for 3 days before the day of surgery and injury, and for 13 days after surgery. After weighing, preservative-free folic acid

(5mg/ml; APP Pharmaceuticals, Inc., USA) was diluted with double-distilled water to 0.125 mg/ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. No nitrous oxide was administered (Supplemental Digital Content fig. 2, 2b, http://links.lww.com/ALN/C22). In protocol 2c, five rats received a single administration of 80% N_2O in 20% oxygen for 4 h immediately before injury. No folic acid was administered (Supplemental Digital Content fig. 2, 2c, http://links.lww.com/ALN/C22). In protocol 2d, four rats received a single administration of 80% N_2O for 4 h immediately before surgery, and 80 μg/kg intraperitoneal folic acid each day for three days before surgery, and for 13 days after surgery (Supplemental Digital Content fig. 2, 2d, http://links.lww.com/ALN/C22). In protocol 2e, nine rats received 40% N_2O in 60% oxygen for 4 h immediately before surgery, and then for 2 h every other day after surgery for 13 days. No folic acid was administered (Supplemental Digital Content fig. 2, 2e, http://links.lww.com/ALN/C22). In protocol 2f, seven rats received 40% N_2O in 60% oxygen for 4 h each day for 3 days before surgery and every other day after surgery for 13 days, and 80 μg/kg intraperitoneal folic acid each day for 3 days before surgery and for 13 days after surgery (Supplemental Digital Content fig. 2, 2f, http://links.lww.com/ALN/C22). In protocol 2g, 16 rats received 80% N_2O in 20% oxygen for 4 h immediately before surgery and then for 2 h every other day after surgery for 13 days. No folic acid was administered (Supplemental Digital Content fig. 2, 2g, http://links.lww.com/ALN/C22). In protocol 2h, 16 rats received 80% N_2O in 20% oxygen for 4 h each day for 3 days before surgery and then for 2 h every other day after surgery for 13 days, and 80 μg/kg intraperitoneal folic acid each day for 3 days before surgery and for 13 days after surgery (Supplemental Digital Content fig. 2, 2h, http://links.lww.com/ALN/C22).

Surgery. To quantify regeneration of spinal axons into a peripheral nerve graft transferred to the cervical spinal cord, adult male Sprague-Dawley rats were anesthetized with ketamine and xylazine, the cervical spinal cord was exposed through a C3 laminectomy, and the dura was opened as previously described[2,3,5] (fig. 1B). With operating microscope guidance, a 0.5-mm-deep injury was made in both posterior columns with a sharp jeweler's forceps, thereby severing primary somatosensory axons ascending the spinal cord. A 1.5-cm autologous sciatic nerve graft harvested from the left hind limb was then implanted at the cervical spinal cord injury site with the distal terminus of the graft sewn to the distal site of spinal cord injury using two to three 10-0 nylon sutures to the dura. The opposite, proximal end of the sciatic nerve graft was tagged with a loosely tied 4-0 silk suture, and freely laid in the subcutaneous space. The wound was closed with 4-0 nylon sutures, and the rats were observed until full recovery from anesthesia. Buprenorphine 0.05 to 0.1 mg/kg (Midwest Veterinary Supply, Inc., USA) was administered subcutaneously as needed for pain.

On the 14th day after injury, the sciatic nerve graft was exposed under ketamine and xylazine anesthesia, sharply cut close to the free end, and a surgical gelfoam 5 mm by 5 mm (Surgicel Johnson-Johnson Ethicon-SARL, LLC, Switzerland) soaked in 5 μl of the preservative-free tracer Fluorogold (Fluorochrome, LLC, USA) was placed to cover the free end of the nerve graft. The fluorescent tracer solely enters axons extending from the dorsal root ganglia that have ascended from the spinal cord injury site into the graft and undergoes rapid retrograde transport to the soma of neurons residing in the dorsal root ganglia. Forty-eight hours later the animals were euthanized.

Dorsal Root Ganglia Section Handling and Analysis. The primary outcome of the percentage of neurons in the dorsal root ganglia that have taken up and transported Fluorogold to their somata provides the index of axon regeneration after injury. After euthanasia, L5 dorsal root ganglia ipsilateral to the sciatic nerve donor site were removed, fixed in 4% paraformaldehyde (Sigma, LLC) at 4°C overnight, and placed the next day in 30% sucrose at room temperature for 3 h incubation (Thermo Scientific D-Sucrose BP220-1, Thermo Scientific, Inc.; fig. 1C). The dorsal root ganglia were then placed in a tissue embedding mold (Polysciences, Inc., USA) with optimum cutting temperature tissue embedding media (10.24% polyvninyl alcohol, 4.25% polyethylene glycerol, 85.50% nonreactive ingredients; Tissue-Tek, Fisher Scientific, Inc., USA), quick-frozen, and placed in a −20°C freezer for 1 to 3 weeks before sectioning. Subsequently, the embedded tissue was cut into 10-um sections with a cryostat (Leica 3050-S, Leica, LLC, Germany) to yield 24 to 35 sections per microscope glass slide (Fisher Scientific, Inc.) stored in a −80°C freezer (Thermo Forma, Fisher Scientific, Inc.) for up to 1 week before scoring. For scoring, the sections were thawed and examined by fluorescent microscopy using an Axioscope 20 fluorescent microscope (Zeiss, LLC, USA) at 20× magnification by two independent observers blinded to treatment and time interval, and the percentage of neurons in the dorsal root ganglia averaged to a single score. The method of Abercombie, in which only cells with visible nucleoli are counted, was used to determine the normative number of neurons observed in rat dorsal root ganglia (*i.e.,* 3,040 on the side ipsilateral to the sciatic nerve injury).[6]

In Vivo Axon Regeneration after Sharp Optic Nerve Injury

Protocol. In protocol 3a, seven control rats received no folic acid or nitrous oxide before or after optic nerve surgery (fig. 1D; Supplemental Digital Content fig. 3, 3a, http://links.lww.com/ALN/C23). In protocol 3b, seven rats received intraperitoneal injections of 80 μg/kg folic acid 3 days before surgery, and then every day until 14 days after surgery (Supplemental Digital Content fig. 3, 3b, http://links.lww.com/ALN/C23). After weighing, preservative-free folic acid (5 mg/ml; APP Pharmaceuticals, LLC, USA) was diluted with double-distilled water to 0.125 mg/

ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. In protocol 3c, eight rats received 80% N_2O in 20% oxygen for 4 h 3 days before surgery, and for 2 h on days 2 and 1 before surgery, and then for 2 h every other day thereafter until 14 days after surgery (Supplemental Digital Content fig. 3, 3c, http://links.lww.com/ALN/C23). In protocol 3d, seven rats received 80% N_2O in 20% oxygen for 4 h 3 days before surgery, and for 2 h on days 2 and 1 before surgery, and then for 2 h every other day thereafter until 14 days after surgery together with intraperitoneal injections of 80 μg/kg folic acid 3 days before surgery, and then every day until 14 days after surgery (Supplemental Digital Content fig. 3, 3d, http://links.lww.com/ALN/C23).

Surgery. Using ketamine and xylazine anesthesia, the optic nerve was exposed through a midline incision and lateral orbital approach superior to the globe, and cut with scissors within 3 mm of the globe.[7,8] One end of an autologous 1.5-cm sciatic nerve graft was attached to the optic nerve stump using two to three 10-0 interrupted nylon sutures, with the distal end of the graft approximated to the optic nerve stump, and the proximal end allowed to lie freely under the skin (fig. 1D). The wound was closed with 4-0 nylon sutures and the rats were observed until full recovery from anesthesia. On the 60th postoperative day, the sciatic nerve graft was exposed under 10:1 ketamine and xylazine anesthesia, trimmed to expose a fresh surface, and a gelfoam saturated in 5 μl of the retrograde preservative-free tracer Fluorogold tracer was applied to the graft at a distance of 1.5 cm from the globe. The wound was closed with 4-0 nylon sutures, and the rats were observed until full recovery from anesthesia. Forty-eight hours later, the animals were anesthetized with ketamine and xylazine, and euthanized by perfusion through the heart with 4% paraformaldehyde.

Retina Handling and Analysis. Each globe was dissected free with forceps and scissors and placed in 4% paraformaldehyde overnight. The following day each globe was transferred to 30% sucrose for 3 h. The cornea was then opened, the lens removed, and the retina extracted through four 0.2-cm incisions that were made along the periphery of each globe. The retinas were then transferred to microscopic glass slides, covered with a coverslip (Thermo Fisher, Inc.), and frozen at −80°C. After thawing, the primary outcome of all retinal ganglion stained cells were counted at 20× under fluorescent microscopy by two independent observers blinded to treatment, and their results per retina were averaged to a single score.

In Vivo Functional Recovery after Blunt Contusion Spinal Cord Injury

Protocol. In protocol 4a, nine rats received no folic acid or nitrous oxide before or after surgery and injury (Supplemental Digital Content fig. 4, 4a, http://links.lww.com/ALN/C24). In protocol 4b, nine rats received daily intraperitoneal folic acid 80 μg/kg in saline beginning 3 days before surgery and injury and continuing daily for 17 days after surgery and injury (Supplemental Digital Content fig. 4, 4b, http://links.lww.com/ALN/C24). After weighing, preservative-free folic acid (5 mg/ml, APP Pharmaceuticals) was diluted with double-distilled water to 0.125 mg/ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. In protocol 4c, eight rats received 80% N_2O in 20% oxygen for 4 h on 3 days before surgery and injury, for 2 h on days 2 and 1 day before surgery and injury, and on every other day for 17 days after surgery and injury (Supplemental Digital Content fig. 4, 4c, http://links.lww.com/ALN/C24). In protocol 4d, seven rats received daily intraperitoneal folic acid 80 μg/kg in saline beginning 3 days before surgery and continuing daily for 17 days, and 80% N_2O in 20% oxygen for 4 h on day 3 before surgery, for 2 h on days 2 and 1 day before surgery, and then every other day for 17 days after surgery (Supplemental Digital Content fig. 4, 4d, http://links.lww.com/ALN/C24).

Surgery. Using ketamine and xylazine anesthesia, the thoracic spinal cord of adult male Sprague–Dawley rats was exposed *via* laminectomy at T9 under aseptic conditions, and a 12.5-gm/cm injury was created using a New York University impactor as previously described.[9,10] The wound was closed with 4-0 nylon sutures and the rats were observed until full recovery from anesthesia. Buprenorphine 0.05 to 0.1 mg/kg was administered subcutaneously as needed for pain.

Behavior Testing. Rats were videotaped for 4 min while ambulating in an open-field environment at baseline 3 days before injury, and weekly intervals thereafter for a period of 5 weeks. Ambulatory function was scored by two blinded and independent observers using the primary outcome of the Basso, Beattie, Bresnahan rating scale, which assigns points for the frequency of occurrence of specific features of normal posture and locomotion, and their results were averaged.[9,10]

Temperature Stability During *In Vivo* Nitrous Oxide Exposure

To test the stability of body temperature during nitrous oxide exposure, hourly rectal temperature measurements were recorded more than a 6-h period of exposure to 80% N_2O in 20% oxygen in six adult male Sprague-Dawley rats.

Statistical Analysis

Data analysis for all protocols was performed using SAS version 9.2 (SAS Institute, Inc., USA). Hypothesis testing was two-sided throughout.

Table 1 provides the number of measured cells used to compare dorsal root ganglia axon length in culture. For statistical analysis of *in vitro* axon regeneration data (protocol 1), pairwise comparisons of axon length at 24 and 48 h among the five groups (*i.e.*, 1: no nitrous oxide [uninjured]; 2: single 70% N_2O exposure [uninjured]; 3: serial 80% N_2O exposures [uninjured]; 4: no nitrous oxide [injured]; and 5: serial 80% N_2O exposures [injured]) were performed using Wilcoxon

Table 1. Dorsal Root Ganglion Axon Length (um) in Culture

Group	No. of Hours	No. of Measured Cells	Mean ± SD	Percent Axons > 300 um
No N_2O (uninjured)	5	87	31 ± 55	1%
	10	76	25 ± 28	0%
	13	143	122 ± 117	10%
	17	155	151 ± 121	12%
	24	155	214 ± 172	31%
	36	53	329 ± 229	49%
	48	46	478 ± 275	76%
Single 70% N_2O (uninjured)	13	135	36 ± 42	0%
	24	105	101 ± 112	6%
	48	192	210 ± 152	24%
Serial 80% N_2O (uninjured)	5	48	29 ± 34	0%
	10	88	36 ± 58	1%
	13	147	50 ± 86	3%
	17	211	64 ± 83	2%
	24	138	100 ± 114	7%
	36	187	107 ± 106	8%
	48	125	115 ± 112	8%
No N_2O (injured)	5	79	68 ± 76	4%
	10	108	187 ± 175	20%
	13	64	234 ± 227	34%
	17	56	390 ± 302	59%
	24	55	501 ± 358	65%
	36	63	522 ± 347	63%
	48	41	825 ± 470	83%
Serial 80% N_2O (injured)	5	118	46 ± 45	0%
	10	150	184 ± 147	22%
	13	166	214 ± 179	26%
	17	74	235 ± 153	28%
	24	154	290 ± 175	47%
	36	165	332 ± 194	47%
	48	114	270 ± 172	39%

N_2O, nitrous oxide.

Table 2. Dorsal Root Ganglion Axon Length in Culture Pairwise Comparison *P* Values

	Axon Length (um)		Percent Axons > 300 um	
Pairwise Group Comparisons	**24 h**	**48 h**	**24 h**	**48 h**
Uninjured no N_2O *vs.* uninjured 70% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Uninjured no N_2O *vs.* uninjured 80% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Uninjured no N_2O *vs.* injured no N_2O	0.003	0.0002	0.0008	0.597
Uninjured 70% N_2O *vs.* uninjured 80% N_2O	0.445	< 0.0001	0.796	< 0.0001
Uninjured 80% N_2O *vs.* injured 80% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Injured no N_2O *vs.* injured 80% N_2O	0.068	< 0.0001	0.246	< 0.0001

N_2O, nitrous oxide.

rank sum test. The proportions of axon lengths greater than 300 um were tested by Fisher exact test. To account for multiple testing, a Bonferroni correction was applied with equally divided overall 10% type I errors among the 24 prespecified comparisons, and a *P* value less than 0.0004 was adopted for all comparisons. Table 2 provides two-sided P values for pairwise comparisons between the five groups.

Table 3 provides the number of animals used to compare the percentage of regenerating dorsal root ganglia neurons after sharp spinal cord injury. For statistical analysis of *in vivo* spinal cord regeneration data (protocol 2), Wilcoxon rank sum tests were performed to compare the percent of regenerating cells. Primary pairwise comparisons comprised: (1) control *versus* folic acid alone; (2) folic acid alone *versus* folic acid plus a single exposure to 80% N_2O; (3) folic acid alone *versus* folic acid plus serial exposures to 40% N_2O; and (4) folic acid alone *versus* folic acid plus serial exposure to 80% N_2O. Secondary pairwise comparisons included: (1) control *versus* single 80%

Table 3. Percentage of Regenerating Dorsal Root Ganglion Neurons after Sharp Spinal Cord Injury

Group	n	Mean ± SD	Minimum	Maximum
Control	14	1.1 ± 0.9	0	2.6
FA	12	13.4 ± 7.1	2.5	23
Single 80% N_2O	5	0.7 ± 0.5	0.2	1.3
Serial 40% N_2O	9	1.3 ± 1.0	0.1	2.9
Serial 80% N_2O	16	0.5 ± 0.8	0	2.4
FA and single 80% N_2O	4	0.6 ± 0.7	0	1.3
FA and serial 40% N_2O	7	0.4 ± 0.5	0	1.3
FA and serial 80% N_2O	16	1.6 ± 1.2	0	3.9

n, number of animals; FA, folic acid 80 μg/kg intraperitoneally; N_2O, nitrous oxide.

Table 4. Number of Regenerating Retinal Ganglion Cells after Sharp Optic Nerve Injury

Group	n	Mean ± SD	Minimum	Maximum
Control	7	895 ± 164	684	1140
FA	7	1277 ± 180	952	1450
FA + 80% N_2O	7	819 ± 155	596	1016

n, number of animals; FA, folic acid 80 μg/kg intraperitoneally; N_2O, nitrous oxide.

N_2O exposure; (2) control *versus* serial 40% N_2O exposure; and (3) control *versus* serial 80% N_2O exposure. A Bonferroni correction for pairwise comparisons was used to test for differences between the groups with a significance level of $P < 0.05$.

Table 4 provides the number of animals used to compare the regenerating retinal ganglion cells after sharp optic nerve injury. For statistical analysis of *in vivo* optic nerve regeneration data (protocol 3), Wilcoxon rank sum tests were performed to compare the number of cells that take up dye in each experimental condition. Pairwise comparisons included: (1) control *versus* folic acid alone and (2) folic acid *versus* folic acid plus nitrous oxide groups. A Bonferroni correction for pairwise comparisons was used to test for differences between the groups with a significance level of $P < 0.05$.

Table 5 provides the number of animals used to compare the spinal cord contusion score estimates for fixed effects. For statistical analysis of functional recovery after contusion spinal cord injury data (protocol 4), a linear mixed model with random intercept and random time effects with the recovery score as the outcome was fitted. Fixed effects in the linear model were: (1) Group (control, folic acid, nitrous oxide, and folic acid plus nitrous oxide); (2) Time (0, 3, 7, 14, 21, 28, and 35 days); and a Group/Time interaction term. A first-order autoregressive matrix was used to model the correlation of the recovery score within the same animal. A significance level of $P < 0.05$ was adopted for all comparisons.

For statistical analysis of temperature during nitrous oxide exposure data, a linear regression model for longitudinal data with a compound-symmetry, intraindividual correlation structure was used to assess temperature constancy more than time. A significance level of $P < 0.05$ was adopted.

No statistical power calculation was conducted before the study. The sample size was based on our previously published experience with the experimental protocols and conduct.[2–4] No outliers were excluded from analysis.

Results

Table 1 provides summary values of experiments conducted to measure *in vitro* dorsal root ganglia axon regeneration at specified hourly intervals after *in vivo* nitrous oxide administration, including the number of longest axons measured per coverslip (n), the mean and SD of the longest axons (um), and the percent of axons greater than 300 um in length. Table 2 provides two-sided *P* values for pairwise comparisons between the five groups. In the uninjured groups (uninj), axon regeneration is double or greater at 24 h and 48 h in the absence of nitrous oxide when compared with either a single 70% N_2O exposure or to serial 80% N_2O exposures ($P < 0.0001$; fig. 2A–C). At 24 h, no difference in inhibition of axonal regeneration in the uninjured groups were observed between a single 70% N_2O exposure and serial 80% N_2O exposures ($P = 0.445$). At 48 h, dorsal root ganglia axon regeneration was halved in the uninjured rats after serial 80% N_2O exposures compared with uninjured rats with a single 70% N_2O exposure.

Table 5. Spinal Cord Contusion Score Estimates for Fixed Effects

Variable	n	Estimate	SEE	*DF*	*t* Value	*P* Value (Pr > |t|)
Control	9	4.53	0.54	194	8.40	< 0.0001
FA	9	5.60	0.54	194	10.40	< 0.0001
N_2O	8	5.33	0.58	194	9.14	< 0.0001
FA + 80% N_2O	7	5.19	0.62	197	8.38	< 0.0001
Time		1.72	0.20	177	8.82	< 0.0001
Time*FA		0.79	0.28	177	2.87	0.005
Time*80% N_2O		0.08	0.30	157	0.27	0.786
Time*FA + 80% N_2O		0.12	0.31	154	0.39	0.696

*indicates the interaction between two variables. *DF*, degrees of freedom; FA, folic acid 80 μg/kg intraperitoneally; n, number of animals; N_2O, nitrous oxide; (Pr > |t|), the *P* values for the effect of the classification variable on the response; SEE, standard error of the estimate.

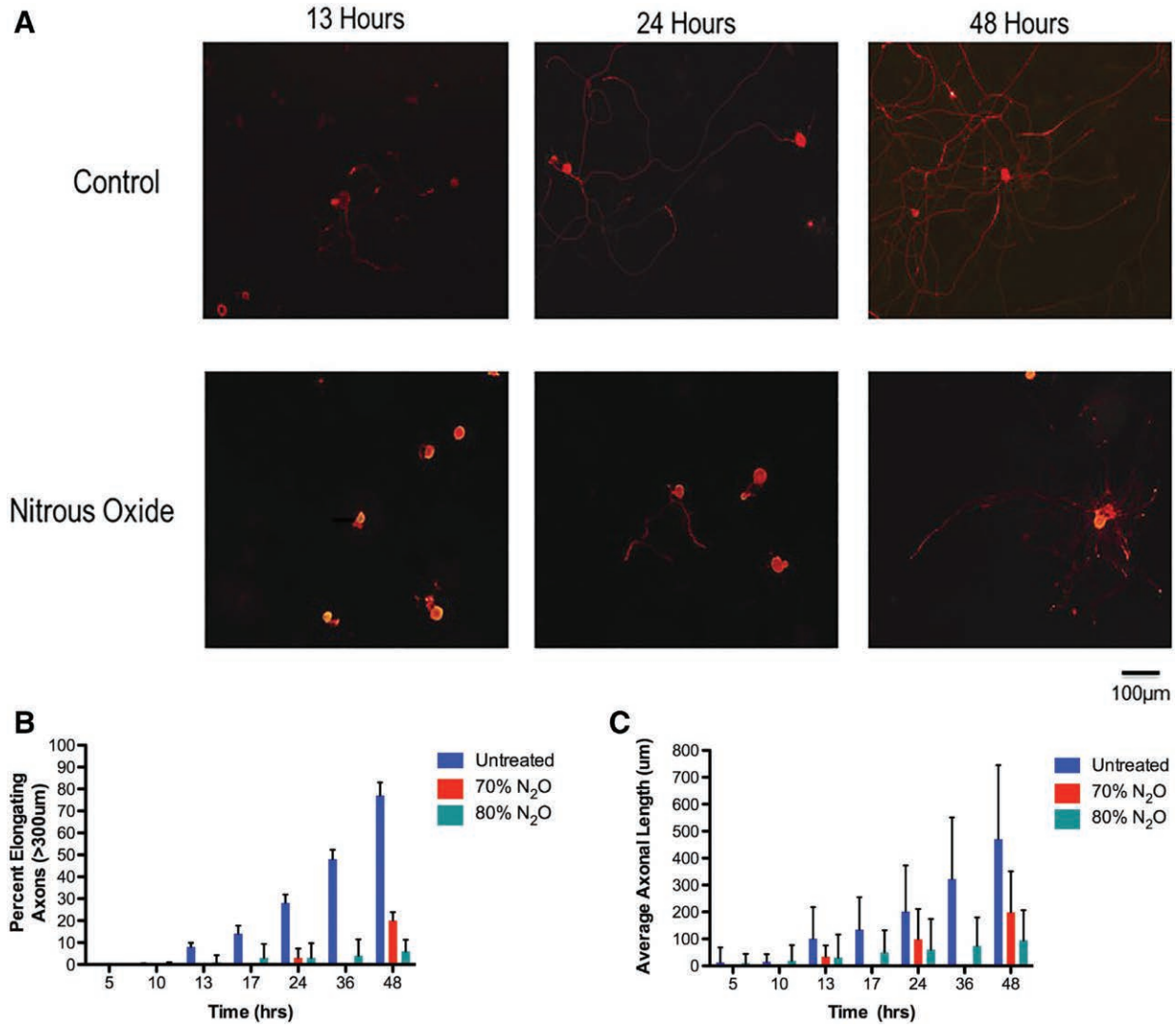

Fig. 2. Representative micrographic images of *in vitro* axon regeneration after *in vivo* nitrous oxide administration (20×). (*A*) Axon growth (*red*) is florid at 48 h in dorsal root ganglia neurons from control animals not exposed to nitrous oxide, but attenuated in dorsal root ganglia neurons from animals exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (protocol 1e). Nitrous oxide impairs the percentage of elongating axons greater than 300 um (*B*), and the average axonal length (*C*) in animals exposed to a single dose of 70% N_2O (*red*; protocol 1b), and in animals exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (*green*; protocol 1e) at 24 and 48 h after culture compared with animals not exposed to nitrous oxide (*blue*). Table 1 provides the number of measured cells used to compare dorsal root ganglia ganglion axon length in culture.

In the absence of nitrous oxide, axon regeneration is greater in the injured group compared with the uninjured group ($P = 0.003$) at 24 h and 48 h. In the presence of serial 80% N_2O exposures, axon regeneration is greater in the injured group compared with the uninjured group ($P < 0.0001$). At 24 h no differences in axonal regeneration in the injured groups were observed between no nitrous oxide exposure and serial 80% N_2O exposures ($P = 0.068$), but at 48 h dorsal root ganglia axon regeneration was markedly lower in the serial 80% N_2O exposure group ($P < 0.0001$). These findings are replicated when the percentage of axons with lengths greater 300 um are tested in all comparisons. The sole exception is between the injured *versus* uninjured group with no nitrous oxide exposure at 48 h, by which time regeneration in the uninjured group is not significantly different from the injured group ($P = 0.597$).

Table 3 provides summary descriptive statistics (*i.e.*, mean, SD, minimum and maximum) for the percentage of dorsal root ganglia neuron axons regenerating after sharp spinal cord injury. Significantly greater spinal cord axon

regeneration was observed in the serial folic acid administration group compared with the control (*i.e.*, no folic acid) group (Z statistic = 4.25, $P < 0.001$). A single 80% N_2O exposure fully reverses the beneficial effect of serial folic acid administration on axon regeneration compared with serial folic acid administration alone (Z statistic = −2.85, $P = 0.004$; figs. 3 and 4). No significant difference in axon regeneration percentages were observed in a comparison between the control group with no serial folic acid administration to the single 80% N_2O exposure with serial folic acid administration group (Z statistic = −0.79, $P = 0.430$). Serial 40% N_2O and serial 80% N_2O exposure reverses the beneficial effect of serial folic acid on axon regeneration (Z statistic = −3.52, $P < 0.001$, and Z statistic = 4.20, $P < 0.001$, respectively). No significant difference in axon regeneration percentages were observed in a comparison between the control group with no serial folic acid administration to the serial 40% N_2O exposure group (Z statistic = 0.28, $P = 0.777$). However, serial 80% N_2O exposure halves axon regeneration compared with control no serial folic acid administration conditions (Z statistic = 2.24, $P = 0.025$).

Table 4 provides summary statistics (*i.e.*, mean, SD, minimum, and maximum) for the number of retinal ganglion cells taking up dye after optic nerve injury. Significantly greater retinal ganglion cell axon regeneration was observed in the serial folic acid administration group compared with the control group (Z statistic = 2.81, $P = 0.005$; figs. 5 and 6). When 80% N_2O was administered together with folic acid, retinal ganglion cell axon regeneration was significantly inhibited (Z statistic = 2.81, $P = 0.005$) compared with serial folic acid administration alone.

Table 5 provides spinal cord contusion score estimates for fixed effects. Table 6 provides pairwise comparisons of spinal cord contusion Basso, Beattie, Bresnahan scores at each time interval with contrast estimates based on the fitted mixed model. The highest Basso, Beattie, Bresnahan scores are observed in the serial folic acid administration alone group (fig. 7). The mixed model demonstrates a significant difference in the overall Basso, Beattie, Bresnahan score trajectories between the four groups (type III test for fixed effects yields chi-square = 332.51, $P < 0.0001$). The Basso, Beattie, Bresnahan score trajectories change significantly more than time (type III test for fixed effects yields chi-square = 330.46, $P < 0.0001$), and the patterns of change

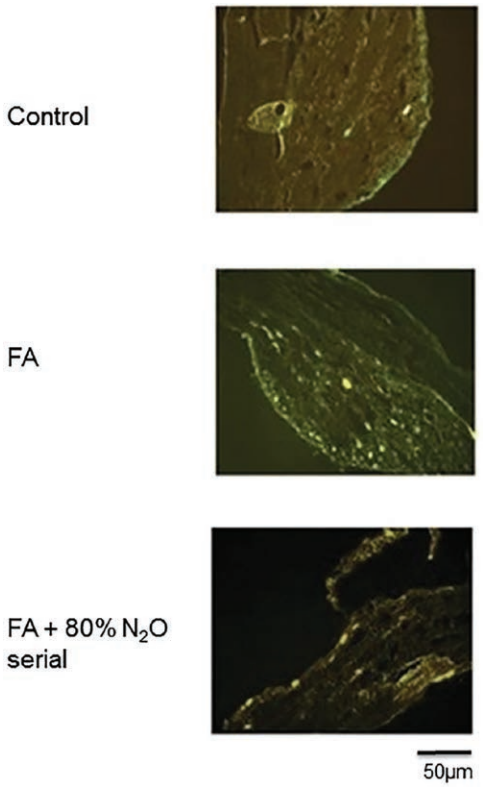

Fig. 3. Representative micrographic images of *in vivo* axon regeneration after sharp spinal cord injury with *in vivo* folic acid ([FA] 80 µg/kg intraperitoneally) and nitrous oxide administration (20×). Folic acid markedly increases the number of dorsal root ganglia neurons that regenerate axons after spinal cord injury and take up a Fluorgold marker for retrograde transport to their cell bodies (*bright green ovals and circles*). Exposure to 80% N_2O for 3 days before injury and every other day thereafter (protocol 2h) reverses the benefit of serial folic acid administration.

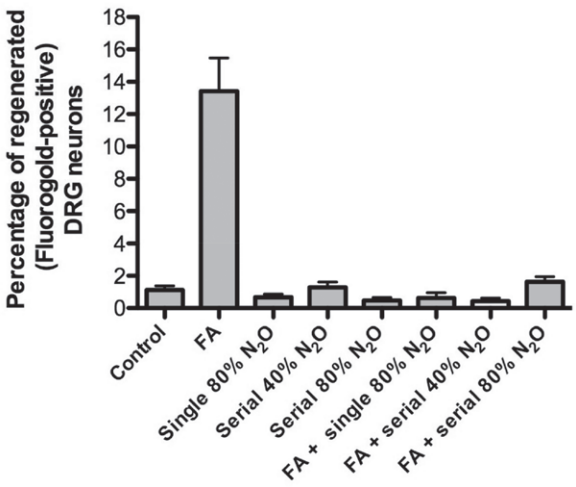

Fig. 4. Folic acid (80 µg/kg intraperitoneally) markedly increases the percentage of regenerated (*i.e.*, Fluorogold-positive) dorsal root ganglia (DRG) neurons (protocol 2b) compared with no folic acid (FA) control (protocol 2a), single 80% N_2O exposure (protocol 2c), serial 40% N_2O exposure (protocol 2e), and serial 80% N_2O exposure (protocol 2g). A single exposure to 80% N_2O (protocol 2d), serial exposure to 40% N_2O (protocol 2f), and serial exposure to 80% N_2O (protocol 2h) reverses the benefit of serial folic acid administration. Table 3 provides the number of animals used to compare the percentage of regenerating dorsal root ganglia neurons after sharp spinal cord injury.

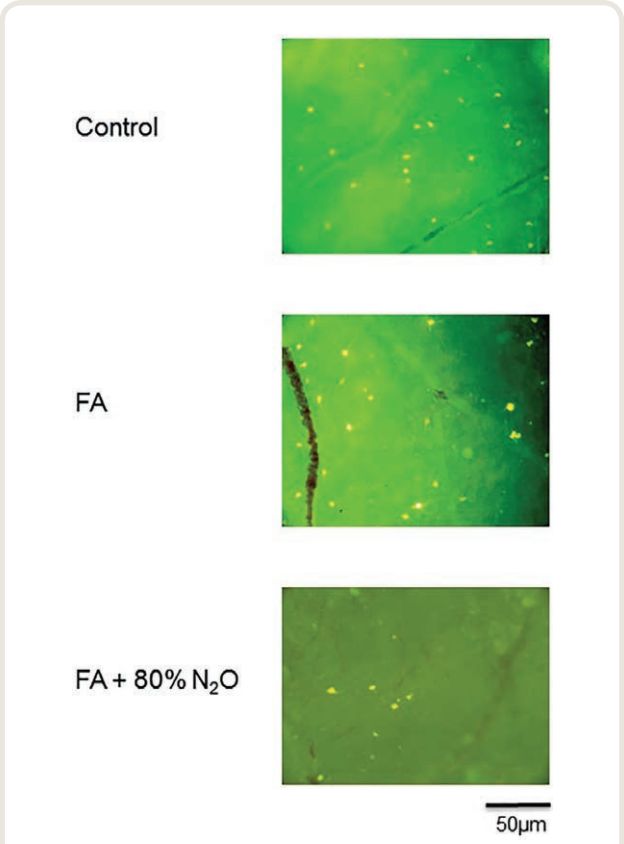

Fig. 5. Representative micrographic images of *in vivo* axon regeneration after sharp optic nerve injury with *in vivo* folic acid ([FA] 80 μg/kg intraperitoneally) and nitrous oxide administration (20×). Folic acid (80 μg/kg intraperitoneally) markedly increases the number of retinal ganglion cells that regenerate axons after optic nerve injury (*bright green circles*). Exposure to 80% N_2O for 3 days before injury and every other day thereafter (protocol 3d) reverses the benefit of serial folic acid administration.

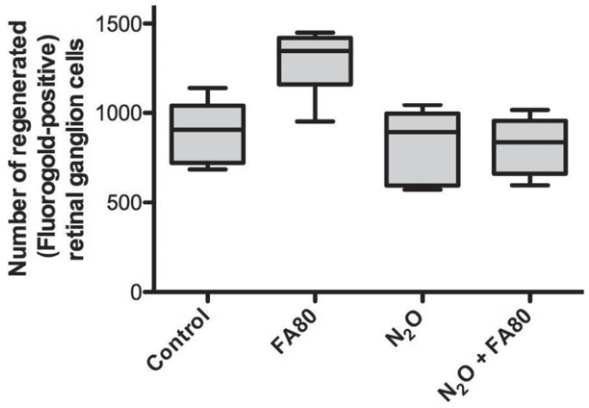

Fig. 6. Folic acid ([FA] 80 μg/kg intraperitoneally) markedly increases the number of (Fluorogold-positive) retinal ganglion cells that regenerate axons (protocol 3b) compared with no folic acid control retinal ganglion cells (protocol 3a), and to serial 80% N_2O exposure retinal ganglion cells (protocol 3c). Serial exposure to 80% N_2O (protocol 3d) reverses the benefit of serial folic acid administration. Table 4 provides the number of animals used to compare the regenerating retinal ganglion cells after sharp optic nerve injury.

are significantly different between the four groups (type III test for fixed effects yields chi-square = 10.15, $P = 0.020$). Results in table 6 indicate that Basso, Beattie, Bresnahan scores in the serial folic acid administration alone group are significantly greater than in the control group at each time interval during the experiment. Basso, Beattie, Bresnahan scores in the serial folic acid administration group are significantly greater than those in the serial 80% N_2O exposure group, and greater than those in the serial folic acid administration and serial 80% N_2O exposure group from week 2 on. No significant differences in Basso, Beattie, Bresnahan scores were observed between the serial 80% N_2O exposure alone, and serial folic acid administration and serial 80% N_2O exposure groups at any time interval in the experiment.

Based on the regression model used, no significant changes in rectal temperature were observed at hourly intervals during 6 h of 80% N_2O exposure (Supplemental Digital Content fig. 5, http://links.lww.com/ALN/C25).

Discussion

Nitrous oxide administered in clinical concentrations and durations during anesthesia is toxic to the dorsal columns of the spinal cord in the absence of nervous system trauma in humans rendered susceptible by genetic and acquired risk factors.[11–30] The effects of nitrous oxide on regeneration of the dorsal columns of the spinal cord and other tissues of the nervous system after sharp and blunt trauma are presently unknown. We report that nitrous oxide in concentrations and durations encountered during clinical anesthesia impairs regeneration of central nervous system axons after peripheral nerve trauma, sharp dorsal spinal cord trauma, sharp optic nerve trauma, and blunt dorsal spinal cord trauma in rodents.

In experiments comprising *in vitro* dorsal root ganglia axon regeneration after *in vivo* nitrous oxide exposure, axon regeneration is greater in the injured sciatic nerve groups compared with the uninjured sciatic nerve groups in the absence and in the presence of nitrous oxide. *In vivo* nitrous oxide exposure inhibits axon regeneration in both the uninjured and injured sciatic nerve groups, with nearly a fourfold difference in axon regeneration observed at 48 h compared with controls after serial nitrous oxide exposure. Of note, both a single nitrous oxide exposure and serial nitrous oxide exposures halve axon regeneration at 24 h in uninjured groups. At 48 h, axon regeneration after a single nitrous oxide exposure in the uninjured group is less than half that in the absence of nitrous oxide, and axon regeneration after serial nitrous oxide exposures in the uninjured group is less than a quarter that in the absence of nitrous oxide. These data indicate that the deleterious effects of

Table 6. Spinal Cord Contusion Score Pairwise Comparisons at Weekly Time Intervals with Contrast Estimates Based on the Fitted Mixed Model

Comparison	Numerator *DF*	Denominator *DF*	*F* Value	*P* Value (Pr > F)
FA *vs.* Control (day −3)	1	208	4.90	0.028
FA *vs.* Control (day 7)	1	187	10.26	0.002
FA *vs.* Control (day 14)	1	48.5	28.17	< 0.0001
FA *vs.* Control (day 21)	1	24.7	37.79	< 0.0001
FA *vs.* Control (day 28)	1	28.8	33.86	< 0.0001
FA *vs.* Control (day 35)	1	39.6	28.16	< 0.0001
FA *vs.* 80% N_2O + FA (day −3)	1	208	1.10	0.295
FA *vs.* 80% N_2O + FA (day 7)	1	187	3.00	0.085
FA *vs.* 80% N_2O + FA (day 14)	1	48.2	10.02	0.003
FA *vs.* 80% N_2O + FA (day 21)	1	26.4	13.99	0.001
FA *vs.* 80% N_2O + FA (day 28)	1	30.5	13.11	0.001
FA *vs.* 80% N_2O + FA (day 35)	1	39.9	11.45	0.002
80% N_2O *vs.* FA (day −3)	1	208	0.83	0.363
80% N_2O *vs.* FA (day 7)	1	186	2.63	0.107
80% N_2O *vs.* FA (day 14)	1	47.3	10.00	0.003
80% N_2O *vs.* FA (day 21)	1	25.4	15.03	0.001
80% N_2O *vs.* FA (day 28)	1	29.1	14.69	0.001
80% N_2O *vs.* FA (day 35)	1	38.4	13.14	0.001
80% N_2O *vs.* N_2O + FA (day −3)	1	208	0.03	0.872
80% N_2O *vs.* N_2O + FA (day 7)	1	186	0.02	0.880
80% N_2O *vs.* N_2O + FA (day 14)	1	47.2	0.01	0.924
80% N_2O *vs.* N_2O + FA (day 21)	1	26.7	0.00	0.984
80% N_2O *vs.* N_2O + FA (day 28)	1	30.4	0.00	0.977
80% N_2O *vs.* N_2O + FA (day 35)	1	38.9	0.00	0.955

DF, degrees of freedom; FA, folic acid 80 μg/kg intraperitoneally; N_2O, nitrous oxide; (Pr > F), the *P* values for the effect of the classification variable on the response.

nitrous oxide on folate-mediated axonal regeneration are neuron specific and do not depend on glial, vascular, or nervous system trophic elements for expression.

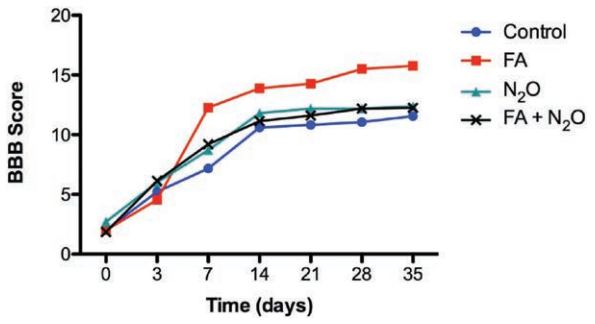

Fig. 7. Functional recovery after blunt contusion spinal cord injury (Basso, Beattie, Bresnahan [BBB]) locomotor scores. Ambulation was scored at baseline 3 days before injury, and at days 7, 14, 21, 28, and 35 after injury. Folic acid ([FA] 80 μg/kg intraperitoneally) improves functional performance scores from day 7 to 35 (protocol 4b; *red squares*) compared with scores from no folic acid control animals (protocol 4a; *blue circles*), and to scores from serial 80% N_2O exposure animals (protocol 4c; *green triangles*). Serial exposure to 80% N_2O (protocol 4d) reverses the benefit of serial folic acid administration (*black x's*). Table 5 provides the number of animals used to compare the spinal cord contusion score estimates for fixed effects.

In experiments comprising *in vivo* dorsal root ganglia axon regeneration after nitrous oxide exposure and sharp spinal cord injury, markedly increased axonal regeneration is observed in the folic acid–treated group compared with the control group as we have previously reported.[2] A single exposure to 80% N_2O is sufficient to fully inhibit the beneficial effects of folic acid on axonal regeneration, as are serial 40% N_2O and 80% N_2O exposures.

In experiments comprising *in vivo* retinal ganglion cell axon regeneration after optic nerve injury and nitrous oxide exposure, serial 80% N_2O administration reverses the regenerative benefits of folic acid. These data indicate that the deleterious effects of nitrous oxide on folate-mediated axonal regeneration are not confined to neurons in the dorsal root ganglia, and that cranial nerves share susceptibility.

In experiments comprising *in vivo* scores of behavioral recovery after direct spinal cord contusion, the marked beneficial effects of folic acid are reversed by coadministration of serial 80% N_2O exposure. These data indicate that the deleterious effects of nitrous oxide on folate-mediated axonal regeneration are not confined to sharp spinal cord injury and have adverse functional consequences in the intact organism in addition to negative histologic outcomes.

The present experiments were not configured to establish a mechanism of neuronal nitrous oxide toxicity. However, reversal of the specific beneficial effects of folic acid with single and serial nitrous oxide exposures, coupled with the well-established property of nitrous oxide to

inactivate methionine synthesis by oxidation of its cobalamin cofactor, points to impaired single carbon metabolism and consequent cellular methyl deficiency as a cause of the observed adverse effects. An important limitation of these data is that we did not examine the effects of nitrous oxide on female rats under identical experimental conditions. To our knowledge, the effects of folic acid and nitrous oxide, the interaction between nitrous oxide and cobalamin, and the capacity for axonal regeneration after injury are shared between the sexes. Future experiments are required to be certain. Although the biophysical interaction between nitrous oxide and cobalamin is identical between species, the time course for nitrous oxide inactivation of methionine synthase is species dependent for reasons yet to be identified. In rats exposed to nitrous oxide, the half-time of hepatic methionine synthase inactivation is 5 min.[31] Recovery takes 3 to 44 days because the vitamin B12 cofactor is irreversibly oxidized and covalently bound to the enzyme. Discontinuation of nitrous oxide does not immediately restore methionine synthase activity since new enzyme must be synthesized to recover from nitrous oxide damage. In humans, the half-life of inactivation in biopsied liver cells is about 45 min.[31] The half-lives of nitrous oxide inactivation and methionine synthase recovery in any tissue of the nervous system *in vivo* are not known in any species. Whether deficiency of methyl substituents necessary for synthesis of neurotransmitters, myelin, DNA and histone methylation and other essential reactions, or accumulation of homocysteine to toxic levels accounts for the pathophysiology alone or in concert is unresolved.

Beyond inter-sex and inter-species differences, a further limitation of the present report is that the data we report are those of first impression, and must therefore await replication by others, and specific clinical correlation before the present results can be safely interpreted. Xenon shares *N*-methyl-D-aspartate glutamate receptor antagonism with nitrous oxide, but has no known effects on cobalamin, methionine synthase and single carbon metabolism. While our experiments comprise specific negative controls, a parallel series of experiments that incorporate equipotent positive control xenon protocols is an attractive next step to begin to discern underlying mechanisms of nitrous oxide neurotoxicity. Future investigations may also comprise direct measures of single carbon pathway enzyme activities, and substrate and product levels in relevant tissues, together with direct measures of nucleic acid, histone and protein methylation.

Surprisingly, nothing is known in any species at present about the effects of nitrous oxide or any other inhaled anesthetic agent on regeneration of nervous system tissues or any other tissue after accidental injury, or after coincidental injury during surgery. Similarly, very little is known about the effects of folic acid, cobalamin, or any other vitamin on regeneration of nervous system tissues after accidental injury, or after coincidental injury during surgery.[2,3] Accordingly, nothing is known about differential susceptibility and resilience to nitrous oxide toxicity, and differential capacity to respond to folate, of different brain regions. These gaps are remarkable in view of the well-recognized deleterious effects of nitrous oxide on nervous tissue that has not been traumatized during clinical anesthesia, and further in view of the substantial proportion of patients who are folate and cobalamin deficient at the extremes of age, and who are surgical candidates.[32,33] The observations reported here suggest that axonal regeneration depends on functional methionine synthase activity, and on the availability of its methyl-donating product methionine for cellular reactions that require methyl additions. When optimal regeneration is desired after neuronal injury, or when surgery itself imperils the nervous system, avoidance of nitrous oxide may be preferred until its comparative safety has been established.

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Hogan: Department of Anesthesiology, University of Wisconsin School of Medicine and Public Health, B6/319 Clinical Sciences Center, 600 Highland Avenue, Madison, Wisconsin 53792. khogan@wisc.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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