Available online at www.sciencedirect.com
SCIENCE DIRECT
Pharmacology, Biochemistry and Behavior 81 (2005) 764 – 768
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Correlation of inbred mouse sensitivity to nitrous oxide antinociception with brain nitric oxide synthase activity following exposure to nitrous oxide

Elisabeth Day Henry [a], Yusuke Ohgami [a,d], Shuang Li [a,b,e], Eunhee Chung [a], Raymond M. Quock [a,b,c,*]

[a]Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, P.O. Box 646534, Pullman, WA 99164-6534, USA
[b]Graduate Program in Pharmacology and Toxicology, Washington State University, Pullman, WA, USA
[c]Center for Integrative Biotechnology, Washington State University, Pullman, WA, USA
[d]Department of School-Nursing, Kyushu Women's Junior College, Fukuoka, Japan
[e]Department of Pharmacology, School of Medicine, University of Washington, Box 357280, Seattle, WA 98195, USA

Received 23 February 2005; received in revised form 25 May 2005; accepted 31 May 2005
Available online 1 July 2005

Abstract

Inhibition of nitric oxide synthase (NOS) antagonizes nitrous oxide (N_2O)-induced antinociception in mice. This study was conducted to compare brain NOS activity in high responding *C57BL/6* mice, low responding *DBA/2* mice and S_5 mice selectively bred for low responsiveness to N_2O. Exposure to 70% N_2O suppressed acetic acid-induced abdominal constrictions in *C57BL/6* mice but not *DBA/2* or S_5 mice. N_2O exposure also elevated NOS activity in brains of *C57BL/6* mice but not *DBA/2* or S_5 mice. The absence of these effects in *DBA/2* or S_5 mice is further support for the hypothesis that nitric oxide (NO) may play a critical role in N_2O-induced antinociception in mice.

Keywords: Nitrous oxide; Antinociception; Nitric oxide synthase; Short-term selective breeding; Mice

1. Introduction

Current evidence indicates that N_2O-induced antinociception in the mouse abdominal constriction model may be secondary to stimulated neuronal release of dynorphin that then activates κ opioid receptors (Quock and Graczak, 1988; Quock et al., 1990; Quock and Mueller, 1991; Branda et al., 2000; Cahill et al., 2000). The activation of these opioid receptors in the periaqueductal gray matter of the brain (Zuniga et al., 1987; Emmanouil et al., 2004), in turn, inhibits GABAergic neuronal influences upon descending pain modulatory systems that involve the activation of adrenergic receptors in the spinal cord (Sawamura et al., 2000; Fujinaga and Maze, 2002).

Another important component of the antinociceptive effect of N_2O appears to be the gaseous neuromodulator nitric oxide (NO). Experiments previously conducted in this laboratory determined that inhibition of NO production in mice reduced their sensitivity to N_2O-induced antinociception in the abdominal constriction test (McDonald et al., 1994; Ishikawa and Quock, 2003b; Li et al., 2004). The present study employed short-term selective breeding as a means of further implicating NO in the mechanism of N_2O-induced antinociception.

Previous research has reported differences in the responsiveness of inbred mouse strains to N_2O-induced antinociception in the acetic acid abdominal constriction test (Quock et al., 1993). The *C57BL/6* mouse strain demonstrated high responsiveness to N_2O, characterized by a significant decrease in abdominal constrictions under 70% N_2O. The

* Corresponding author. Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, P.O. Box 646534, Pullman, WA 99164-6534, USA. Tel.: +1 509 335 5956; fax: +1 509 335 5902.
E-mail address: quockr@wsu.edu (R.M. Quock).

0091-3057/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2005.05.013

DBA/2 mouse strain responded poorly to N_2O, showing very little change in abdominal constrictions. In a recent preliminary report, we found that exposure to 70% N_2O caused a 40% elevation in whole brain NOS activity in *C57BL/6* mice but only a statistically insignificant 10% increase in *DBA/2* mice (Ishikawa and Quock, 2003a). The present research shows that exposure to N_2O increases NOS enzyme activity in whole brain and cerebellum and decreases NOS activity in the corpus striatum of *C576/BL* mice but not DBA or a line of mice that was selectively bred for low responsiveness to N_2O antinociception.

2. Methods

2.1. Animals

Adult male DBA ($n=15$) and C57 male mice ($n=14$) were obtained from the Jackson Laboratory (Bar Harbor, Maine). S_5 mice ($n=14$) with poor responsiveness to N_2O-induced antinociception were the product of a short-term selective breeding program similar to that described by Belknap et al. (Belknap et al., 1997). Breeding began with low-responding male and female mice from the F_2 generation derived from DBA and C57 progenitors (Mueller et al., 2004). Each generation was screened for responsiveness to N_2O-induced antinociception, and the male and female mice with the poorest responsiveness were mated to produce the next generation. Breeding was ended following the F_7 (or S_5) generation.

These experiments were approved by an institutional animal care and use committee and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). All measures to minimize pain or discomfort were taken by the investigators.

2.2. Antinociceptive testing

Antinociceptive responsiveness to N_2O was assessed by the abdominal constriction test. At 7–8 weeks of age, mice were treated intraperitoneally with 0.6% acetic acid (0.1 ml/10 g body weight); exactly 5 min later, the number of abdominal constrictions—lengthwise stretches of the torso with concave arching of the back—in each animal was counted for a 6-min period while in a Plexiglas exposure chamber (20 cm W × 35 cm L × 15 cm H) open to room air. One week later, mice were again treated with acetic acid, but this time housed in a closed chamber containing an atmosphere of 70% N_2O in oxygen (O_2). Previous studies showed that mice typically regained their sensitivity to acetic acid-induced abdominal constrictions within this period of time.

2.3. Delivery of nitrous oxide/oxygen

A mixture of 70% N_2O, U.S.P. and 30% O_2, U.S.P. (A&L Welding, Spokane, Washington) was delivered into the chamber at a total inflow rate of 10 l/min using a portable N_2O/O_2 dental sedation system (Porter, Hatfield, Pennsylvania). A POET II anesthetic monitoring system (Criticare, Milwaukee, Wisconsin) was used to verify that desired N_2O/O_2 concentrations had been attained. Exhausted gas was vented to a nearby fume hood.

2.4. NOS assay

Mice were decapitated after 15 min exposure to 70% N_2O or room air (as control). The brains were quickly removed and placed on an ice-cold Petri dish. The brain was cut in half along the midline. One brain half was used to determine NOS activity level per milligram protein. The other brain half was dissected to measure NOS activity in hippocampus, cerebellum, amygdala, midbrain and corpus striatum. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed.

NOS activity was assayed by measuring the Ca^{2+}-dependent conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline (Huang et al., 1993). On the day of the assay, tissue samples were sonicated in 10 volumes (wt/vol) of 50 mM Tris·HCl (pH 7.4) buffer containing 1.0 mM ethylenediamine tetraacetic acid (EDTA) and 1.0 mM ethyleneglycol tetraacetic acid (EGTA) (homogenization buffer). After centrifugation (12,000 rpm for 20 min at 4 °C), 20 μl supernatant was added to 40 μl 50 mM Tris·HCl (pH 7.4) buffer containing 1.0 mM NADPH, 3.0 μM BH_4, 1.0 μM FAD, 1.0 μM FMN, 1.25 mM $CaCl_2$, and 1.25 μCi/ml [^{14}C]L-arginine (specific activity: 348 mCi/mmol, Amersham Biosciences, Piscataway, New Jersey) and incubated for 30 min at 37 °C. The reaction was terminated by addition of 400 μl stop buffer containing 50 mM HEPES (pH 5.5) and 5.0 mM EDTA. Then the reaction mixture was applied onto a chromatographic column containing 40 mg Dowex AG50WX-8 resin (Bio-Rad, Hercules, California) for separation of [^{14}C]L-citrulline from the unreacted [^{14}C]L-arginine by cation-exchange chromatography and collected into a scintillation vial. Thereafter, the samples were counted for the amount of radioactivity using a model A2500 liquid scintillation counter (Packard Instrument Company, Meriden, Connecticut). The protein content of the supernatant was determined using the bicinchoninic acid (BCA) method and a commercially available assay kit (Pierce Chemical Company, Rockford, Illinois) with bovine albumin as a standard. NOS enzyme activity was expressed in terms of picomole per milligram protein per minute.

2.5. Statistical analysis of data

The responsiveness of each strain to 70% N_2O was determined by Dunnett's *t*-test. Differences in NOS activity levels of N_2O- and room air-exposed animals were analyzed by paired Student's *t*-test.

3. Results

When exposed to room air, *C57BL/6* mice exhibited 10.0±1.6 (mean±S.E.M.) abdominal constrictions over 6 min. One week later, when exposed to 70% N_2O, these mice exhibited 2.8±0.8 abdominal constrictions over the same time resulting in a 72.4±7.1% antinociceptive response. By comparison, *DBA/2* mice exhibited 7.0±1.1 abdominal constrictions under room air and 6.4±1.2 abdominal constrictions under N_2O (8.6±17.4% antinociceptive response), and S_5 mice strains responded with 7.9±1.4 abdominal constrictions under room air and 8.2±1.7 abdominal constrictions under N_2O (−3.6±20.7% antinociceptive response) (Fig. 1).

Exposure to 70% N_2O resulted in significant increases in NOS activity in whole brains and cerebella of *C57BL/6* mice compared to activity levels in room air-exposed mice. NOS activity in the corpus striatum of *C57BL/6* was reduced by 70% N_2O, while NOS activities in the midbrain and hippocampus were not significantly different between room air and 70% N_2O (Fig. 2). Results showed that N_2O exposure did not increase NOS activity in either whole brain or regional brain of *DBA/2* or S_5 mice. On the contrary, S_5 mice exhibited a decrease in NOS activity in whole brain and all brain regions examined, excluding the brainstem.

4. Discussion

To our knowledge, there has been only one earlier series of studies involving selective breeding of mice for differences in sensitivity to N_2O. Mice were bred through ten generations to produce offspring that were highly susceptible or highly resistant to N_2O anesthesia (as determined by loss of the righting reflex) (Koblin et al., 1980). Animals that were highly resistant to N_2O were cross-resistant to other inhalation anesthetics and to ethanol as well (Koblin et al., 1982a), were more susceptible to barbiturate-induced hypnosis (Koblin et al., 1984), although N_2O-resistant mice were more susceptible to convulsant drugs (Koblin et al., 1982b). The difference in ED50 values for anesthesia between N_2O-susceptible and resistant mice appeared to be inversely related to the lipid solubility of the anesthetic, i.e., very small difference between the mice for highly lipid-soluble anesthetics and very large difference between the mice for poorly lipid-soluble anesthetics (Koblin et al., 1982a). In contrast, another measure of anesthesia (responsiveness to tail-clamp pinch)—which, in all likelihood, reflects antinociception rather than anesthesia—showed a comparable separation in ED50 values for each of the anesthetics tested in both N_2O-susceptible and -resistant mice (Koblin et al., 1982a).

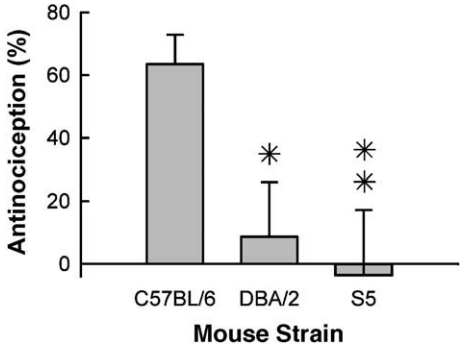

Fig. 1. N_2O-induced suppression of acetic acid abdominal constrictions in *C57BL/6* (C57), *DBA/2* (DBA) and S_5 mice. See text for calculation of % antinociception. Significance of difference: $^{**}p<0.01$, compared to *C57BL/6* mice (Dunnett's *t*-test).

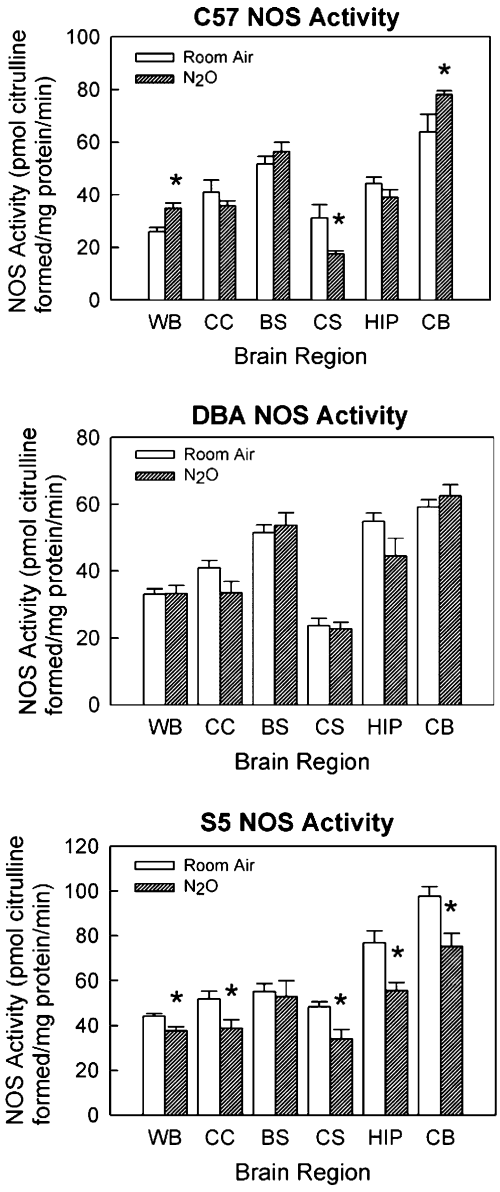

Fig. 2. Effects of N_2O exposure on NOS activity in *C57BL/6* (C57), *DBA/2* (DBA) and S_5 mice. Abbreviations: WB, whole brain; CC, cerebral cortex; BS, brainstem; CS, corpus striatum; HIP, hippocampus; CB, cerebellum. Significance of difference: $^{*}p<0.05$, compared to room air control of the same strain (paired *t*-test).

Previous research has demonstrated that N_2O-induced antinociception in mice and rats is NO-dependent. Pretreatment of mice with the NOS-inhibitors L-N^G-nitro arginine (L-NOARG), L-N^G-nitro arginine methyl ester (L-NAME) and L-N^G-monomethyl nitro arginine (L-NMMA) all resulted in marked attenuation of the antinociceptive response of mice to N_2O in the abdominal constriction test (McDonald et al., 1994). A subsequent study reported that N_2O-induced antinociception was also sensitive to antagonism by *S*-methyl-L-thiocitrulline (SMTC), a putatively selective inhibitor of neuronal NOS, and also by higher doses of L-N^5-(1-iminoethyl)ornithine (L-NIO), which is approximately 8-fold more potent against eNOS than nNOS and 4-fold more potent against eNOS than iNOS (Rees et al., 1990; McCall et al., 1991) but has been shown to lose the selectivity at higher doses (Li et al., 2003). Pretreatment with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), which selectively inhibits inducible NOS, was ineffective in antagonizing N_2O-induced antinociception. In the rat hot plate test, the antinociceptive response to 70% N_2O was antagonized in dose-related manner by i.c.v. pretreatment with L-NOARG or L-NAME; this antagonism was again reversed by L-arginine but not D-arginine (McDonald et al., 1994).

Based on the above observations, we have suggested that stimulated neuronal release of endogenous opioid peptides may be an NO-dependent process (Hara et al., 1995). NO targets the metal centers of metalloenzymes (Bredt, 1996), of which the best characterized is soluble guanylyl cyclase (sGC). This enzyme may regulate the neuronal release of opioid peptide.

Previous research has reported differences in the responsiveness of inbred mouse strains to N_2O-induced antinociception in the acetic acid abdominal constriction test (Quock et al., 1993). The *C57BL/6* mouse strain demonstrated high responsiveness to N_2O, characterized by a significant decrease in abdominal constrictions under 70% N_2O. The *DBA/2* mouse strain responded poorly to N_2O, showing very little change in abdominal constrictions. In a recent preliminary report, we found that exposure to 70% N_2O caused a 40% elevation in whole brain NOS activity in *C57BL/6* mice but only a statistically insignificant 10% increase in *DBA/2* mice (Ishikawa and Quock, 2003a). The present research shows that exposure to N_2O increases NOS enzyme activity in whole brain and cerebellum and decreases NOS activity in the corpus striatum of *C576/BL* mice; however, there were no changes in NOS activity in DBA mice and significant decreases in whole brain and regional brain levels of NOS activity in S_5 mice.

Previous pharmacogenetic studies from our laboratory have identified quantitative trait loci (QTL) or are regions of the genome containing genes that are highly associated with responsiveness to N_2O-induced antinociception (Quock et al., 1996). One significant QTL was located on proximal Chromosome 2 near the marker *D2Mit91* ($p = 5 \times 10^{-5}$), and the other was located on distal Chromosome 5 near *D5Mit409* ($p = 1.3 \times 10^{-4}$). It is worth noting that our Chromosome 5 QTL maps close to *Nos1* on Chromosome 5 (65 cM), which is the gene that encodes neuronal NOS (www.informatics.jax.org). This QTL has a peak LOD at 84 cM, but the confidence interval clearly encompasses the *Nos1* locus. While these findings pertain specifically to N_2O-induced antinociception, they are in general agreement with the claim that genetic control of resistance or susceptibility to N_2O anesthesia probably involves multiple genes (Koblin and Eger, 1981).

In the present study, *C57BL/6* mice responded to N_2O with antinociception and increased NOS activity, while *DBA/2* and S_5 mice responded to N_2O with poor antinociception and no change or a slight reduction in NOS. The co-segregation of the traits for poor antinociception and failure to elevate NOS activity is further evidence of a possible correlation between elevation in NOS activity in response to N_2O and an antinociceptive response to N_2O.

Acknowledgements

This research was supported by NIH Grants DA-10047 and DA10343. We are grateful to Dr. John K. Belknap (Oregon Health and Science University, Portland, Oregon) for his counsel during the short-term selective breeding program.

References

Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ. Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. Behav Genet 1997;27:55–66.

Branda EM, Ramza JT, Cahill FJ, Tseng LF, Quock RM. Role of brain dynorphin in nitrous oxide antinociception in mice. Pharmacol Biochem Behav 2000;65:217–22.

Bredt DS. Targeting nitric oxide to its targets. Proc Soc Exp Biol Med 1996;211:41–8.

Cahill FJ, Ellenberger EA, Mueller JL, Tseng LF, Quock RM. Antagonism of nitrous oxide antinociception in mice by intrathecally administered opioid peptide antisera. J Biomed Sci 2000;7:299–303.

Emmanouil DE, Ohgami Y, Chung E, Han S, Quock RM. Nitrous oxide (N_2O) antinociception in the mouse abdominal constriction test is mediated by opioid receptors in the periaqueductal gray region of the brain. Proc West Pharmacol Soc 2004;47:151.

Fujinaga M, Maze M. Neurobiology of nitrous oxide-induced antinociceptive effects. Mol Neurobiol 2002;25:167–89.

Hara S, Kuhns ER, Ellenberger EA, Mueller JL, Shibuya T, Endo T, et al. Involvement of nitric oxide in intracerebroventricular β-endorphin-induced neuronal release of methionine–enkephalin. Brain Res 1995;675:190–4.

Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC. Targeted disruption of the neuronal nitric oxide synthase gene. Cell 1993;75:1273–386.

Ishikawa M, Quock RM. N_2O stimulates NOS enzyme activity in *C57BL/6* but not *DBA/2* mice. Brain Res 2003a;976:262–3.

Ishikawa M, Quock RM. Role of nitric oxide synthase isoforms in nitrous oxide antinociception in mice. J Pharmacol Exp Ther 2003b;306:484–9.

Koblin DD, Eger II EI. Cross-mating of mice selectively bred for resistance or susceptibility to nitrous oxide anesthesia: potencies of nitrous oxide in offspring. Anesthesiol 1981;60:646–8.

Koblin DD, Dong DE, Deady JE, Eger II EI. Selective breeding alters murine resistance to nitrous oxide without alteration in synaptic membrane lipid composition. Anesthesiology 1980;52:401–7.

Koblin DD, Deady JE, Eger II EI. Potencies of inhaled anesthetics and alcohol in mice selectively bred for resistance and susceptibility to nitrous oxide anesthesia. Anesthesiology 1982a;56:18–24.

Koblin DD, O'Connor B, Deady JE, Eger II EI. Potencies of convulsant drugs in mice selectively bred for resistance or susceptibility to nitrous oxide. Anesthesiol 1982b;56:25–8.

Koblin DD, Lurz FW, Eger II EI. Potencies of barbiturates in mice selectively bred for resistance or susceptibility to nitrous oxide anesthesia. Anesth Analg 1984;63:35–9.

Li S, Ohgami Y, Dai Y, Quock RM. Antagonism of nitrous oxide-induced behavior in mice by pharmacologic disruption of endogenous nitric oxide function. Psychopharmacology 2003;166:366–72.

Li S, Bieber AJ, Quock RM. Antagonism of nitrous oxide antinociception in mice by antisense oligodeoxynucleotide directed against neuronal nitric oxide synthase enzyme. Behav Brain Res 2004;152:361–3.

McCall TB, Feelisch M, Palmer RM, Moncada S. Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br J Pharmacol 1991;102(1):234–8 [Jan].

McDonald CE, Gagnon MJ, Ellenberger EA, Hodges BL, Ream JK, Tousman SA, et al. Inhibitors of nitric oxide synthesis antagonize nitrous oxide antinociception in mice and rats. J Pharmacol Exp Ther 1994;269:601–8.

Mueller JL, Ellenberger EA, Vaughn LK, Belknap JK, Quock RM. Detection and mapping of quantitative trait loci (QTLs) that determine responsiveness of mice to nitrous oxide (N_2O) antinociception. Neurosci 2004;123:743–9.

Quock RM, Graczak LM. Influence of narcotic antagonist drugs upon nitrous oxide analgesia in mice. Brain Res 1988;440:35–41.

Quock RM, Mueller J. Protection by U-50,488H against beta-chlornaltrexamine antagonism of nitrous oxide antinociception in mice. Brain Res 1991;549:162–4.

Quock RM, Best JA, Chen DC, Vaughn LK, Portoghese PS, Takemori AE. Mediation of nitrous oxide analgesia in mice by spinal and supraspinal kappa-opioid receptors. Eur J Pharmacol 1990;175:97–100 [corrigendum 187: 564].

Quock RM, Mueller JL, Vaughn LK. Strain-dependent differences in responsiveness of mice to nitrous oxide antinociception. Brain Res 1993;614:52–6.

Quock RM, Mueller JL, Vaughn LK, Belknap JK. Nitrous oxide antinociception in *BXD* recombinant inbred mouse strains and identification of quantitative trait loci. Brain Res 1996;725:23–9.

Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. Br J Pharmacol 1990;101(3):746–52 [Nov].

Sawamura S, Kingery WS, Davies MF, Agashe GS, Clark JD, Kobilka BK, et al. Antinociceptive action of nitrous oxide is mediated by stimulation of noradrenergic neurons in the brainstem and activation of α_{2B} adrenoceptors. J Neurosci 2000;20:9242–51.

Zuniga J, Joseph S, Knigge K. Nitrous oxide analgesia: partial antagonism by naloxone and total reversal after periaqueductal gray lesions in the rat. Eur J Pharmacol 1987;142:51–60.