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Detection and Mapping of Quantitative Trait Loci that Determine Responsiveness of Mice to Nitrous Oxide Antinociception

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Abstract

Exposure to 70% N_2O evokes a robust antinociceptive effect in C57BL/6 (B6) but not in DBA/2 (D2) inbred mice. This study was conducted to identify quantitative trait loci (QTL) in the mouse genome that might determine responsiveness to N_2O. Offspring from the F_2 generation bred from B6 and D2 progenitors exhibited a broad range of responsiveness to N_2O antinociception as determined by the acetic acid-induced abdominal constriction test. QTL analysis was then used to dissect this continuous trait distribution into component loci, and to map them to broad chromosomal regions. To this end, 24 spleens were collected from each of the following four groups: male and female F_2mice responding to 70% N_2O in oxygen with 100% response (high-responders); and male and female F_2 mice responding with 0% response (low-responders). Genomic DNA was extracted from the spleens and genotyped with simple sequence length polymorphism MapPairs markers. Findings were combined with findings from the earlier QTL analysis from BXD recombinant inbred mice [Brain Res 725 (1996) 23]. Combined results revealed two significant QTL that influence responsiveness to nitrous oxide on proximal chromosome 2 and distal chromosome 5, and one suggestive QTL on midchromosome 18. The chromosome 2 QTL was evident only in males. A significant interaction was found between a locus on chromosome 6 and another on chromosome 13 with a substantial effect on N_2O antinociception.

Keywords

nitrous oxide; antinociception; quantitative trait loci; mice

The simple inorganic pharmacological gas N_2O produces a prominent antinociceptive effect in experimental animals as well as clinical analgesia in human patients (Finck, 1985). Though long thought to be due to a nonspecific depression of CNS function (Sonnenschein et al., 1948), this drug effect of N_2O is now known to involve the activation of endogenous opioid systems (Quock and Vaughn, 1995; Fujinaga and Maze, 2002). This was clearly shown by Berkowitz and his associates who found that N_2O antinociception in mice and rats was antagonized by the opioid receptor blocker naloxone (Berkowitz et al., 1976, 1977) and that morphine-tolerant animals were cross-tolerant to N_2O (Berkowitz et al., 1977, Berkowitz et al., 1979). The first elaboration of these findings using subtype-selective opioid receptor blockers was conducted by our laboratory and demonstrated that in the mouse abdominal constriction test, N_2O antinociception is specifically mediated by κ opioid receptors (Quock et al., 1990). This was verified by a receptor protection experiment in which co-administration of a κ opioid ligand protected N_2O antinociception from antagonism by a non-selective opioid receptor alkylating antagonist, β-chlornaltrexamine (Quock and Mueller, 1991). More recent studies utilizing rabbit antisera against opioid peptides demonstrated that these κ opioid receptors were activated by dynorphin fragments during N_2O antinociception (Branda et al., 2000, Cahill et al., 2000).

In research utilizing inbred mouse strains, we discovered that, compared with the C57BL/6 (B6) strain, DBA/2 (D2) mice were significantly less responsive to N_2O antinociception (Quock et al., 1993). To further investigate the mechanism of N_2O antinociception, more recent studies have utilized a pharmacogenetic approach to identify quantitative trait loci (QTL) in the mouse genome that might determine responsiveness to N_2O. A study conducted in BXD recombinant inbred mice derived from the B6 and D2 strains identified some provisional QTL that determine responsiveness to N_2O (Quock et al.,

1996). The present study was conducted in a large population of the F_2 generation bred from the B6 and D2 progenitors to confirm the identities of these QTL.

Experimental procedures

Animals

A breeding colony was established with male and female B6D2 F_1 mice (Charles River Laboratories, Charles River, MA, USA). Nearly 500 offspring from the F_2 generation were bred and housed in an AAALAC-accredited temperature- and humidity-regulated vivarium with a 12-h light/dark cycle. Food and water were available *ad libitum*.

All experiments were approved by an institutional animal care and use committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Antinociceptive testing

Antinociceptive responses were assessed using the abdominal constriction test. At 7–8 weeks of age, mice were treated intraperitoneally with 0.1 ml per 10 g body weight of 0.6% acetic acid; 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 in an atmosphere of air from a compressed air cylinder. One week later, they were again tested for responsiveness to 0.6% acetic acid but this time in an atmosphere of 70% N_2O in oxygen (O_2). The degree of antinociception (inhibition of abdominal constrictions) produced by N_2O in each animal was calculated as:

% antinociception = 100

$$\frac{\#\ constrictions\ in\ compressed\ air - \#\ constrictions\ in\ N_2O}{\#\ constrictions\ in\ compressed\ air}$$

Exposure to N_2O

Groups of five mice each were placed inside a Plexiglas exposure chamber (20 cm W×35 cm L×15 cm H) immediately following treatment with acetic acid. A mixture of 70% N_2O, U.S.P. and 30% O_2, U.S.P. (Rockford Industrial Gas, Rockford, IL, USA) was delivered into the chamber via a length of polyethylene tubing during a 5-min filling time using a portable N_2O/O_2 dental sedation system (Porter, Hatfield, PA, USA). A POET II anesthetic monitoring system (Criticare, Milwaukee, WI, USA) was used to ascertain that desired N_2O/O_2 atmospheres had been attained. Exhausted gas was vented to a fume hood via a second length of polyethylene tubing. Control mice were exposed to compressed air, U.S.P. delivered into the chamber at the same inflow rate (10 l/min).

QTL analysis

Following antinociceptive testing, mice were killed. The spleens were dissected from all mice, stored in cryovials containing 0.5 ml physiological saline and saved at −20 °C. Genomic DNA was extracted from the spleens by Nucleic Acid Purifications (Huntsville, Alabama). Genotyping was accomplished by Research Genetics Inc. (Huntsville, AL, USA) with simple sequence length polymorphism MapPairs markers (Jacob et al., 1995).

Selective genotyping was used where only the extreme high and low scoring tails of the trait distribution were genotyped, a total of 96 of 493 mice. The proportion of genotyped mice of 19.5% (both tails combined) contained 64% of the QTL information as do all 493 mice, but required only about one-fifth of the genotyping cost (Lander and Botstein, 1989, Darvasi and Soller, 1992). A total of 77 markers were genotyped, a process that determines which genotype each mouse possesses at each marker. The average interval between markers was 19 cM with no interval greater than 33 cM. In an F_2 mouse, the genotype at any one marker is either homozygous B6, homozygous D2 or heterozygote. Several markers were genotyped on each chromosome, and any marker whose genetic variation significantly covaried with trait variation provided evidence for the presence of a QTL near the marker. The marker genotype and trait data were analyzed for QTL and for interactions between loci (epistasis) using the Pseudomarker 0.9 program package written for the MATLAB (Mathworks Inc., Natick, MA, USA) programming environment (Sen and Churchill, 2001; obtained from www.jax.org/research/churchill) using default settings except where noted otherwise. This program uses Monte Carlo computer sampling to estimate the most likely values of unknown parameters (QTL genotypes, locations, missing data) using Bayesian methods. Sixty-four imputations (samplings) of unknown genotypes based on the observed marker data were made at 5 cM intervals, which approximates interval mapping at this resolution. This was followed by 2.5 cM mapping for the more interesting chromosomes. This program package analyzes the phenotypic effect of each marker or marker interval taken singly (MAINSCAN), but also the phenotypic effects of pairs of markers or intervals taken jointly (PAIRSCAN) for their effects on the trait. The latter allows a genome-wide search for epistasis, which are interactions between loci.

The criterion for statistical significance was based on a genome-wide $P<0.05$ estimated by permutation tests on F_2 data. This method takes the trait data for individual mice, and reassigns them at random to the genotypes 1000 times. This simulates the null hypothesis that there are no QTL anywhere in the genome, thus any apparent QTL are, by definition, false positives (Churchill and Doerge, 1994). The 5% highest LOD scores were identified and the threshold, which separates them from the other 95%, was taken as the genome-wide empirical significance threshold for this data set. This value was $P=0.0002$ for single markers or intervals (MAINSCAN). The threshold for a suggestive QTL was $P=0.005$, which allows an average of one false positive QTL genome-wide (Lander and Kruglyak, 1995). These P values correspond to LOD 3.7 and 2.3, respectively, $df=2$, based on the expression LOD (logarithm of the odds)$=-\log_{10}(P)$. For the BXD data, we used $P=0.0002$ as our significant criterion determined by permutation tests carried out in the same manner as the F_2.

The P values from both BXD and F_2 data for the same or similar markers were combined using Fisher's method for combining P values from independent experiments testing the same hypothesis (Sokal and Rohlf, 1995; Belknap et al., 1996). This was done only when the direction of QTL effect, or which allele was associated with higher trait scores, was the same in both BXD and F_2 data. For the combined results, we used the average of the F_2 and BXD thresholds, or $P=0.0002$ (LOD 3.7) for significant, and $P=0.005$ (LOD 2.3) for suggestive.

For tests of epistasis (PAIRSCAN), we required that the combined (or full model) effects on the trait of a marker pair, which reflects the main effects of both markers plus their interaction, exceed the threshold for significance (5% genome-wide error rate) estimated by a permutation test carried out using the *Pseudomarker* PAIRSCAN program on F_2 data. In this study, the threshold was set at LOD 7.9 ($df=8$, $P=1.5\times10^{-5}$) based on 500 permutations. When the combined effects of a marker pair were significant, we then tested for their interaction at a significance threshold of $P<0.01$ (Sen and Churchill,

2001). This approach represents a conditional search for epistasis because only marker pairs attaining significance for their joint effects on a trait (the condition) are tested for possible interactions. This greatly reduces the number of pairs tested for interactions (only two in this study), and thus the rate of false positive interactions compared with an unconditional search (Hood et al., 2001).

For those QTL attaining either suggestive or significant status (three QTL), we looked at the F_2 data to determine whether there were gender differences in QTL effect magnitude. Because we looked at only three chromosomal regions, the threshold for significance for the gender difference was P=0.05/3, or 0.0167. This is equivalent to a LOD score difference of 1.8 between the two genders. Because the BXD data (Quock et al., 1996) were collected on males only, only the F_2 was useful for assessing gender differences.

Results

A total of 493 F_2 mice were tested for antinociceptive responsiveness to 70% N_2O. The distribution of their responsiveness to N_2O is shown in Fig. 1. When genders were combined, roughly one quarter of the F_2 mice exhibited less than 10% antinociceptive response, which includes hyperalgesic response, and roughly one quarter of the F_2 mice exhibited 90–100% antinociceptive response. When only the 220 male F_2 mice were considered, roughly 22% showed less than 10% antinociceptive response, while roughly 32% showed 90–100% antinociceptive response. When only the 273 female F_2 mice were considered, roughly 31% showed less than 10% antinociceptive response, while 22% showed 90–100% antinociceptive response. However, to insure equal representation for QTL analysis, the mice chosen for genotyping (N=96) were evenly split between the high and low tails of the distribution and also between the two genders.

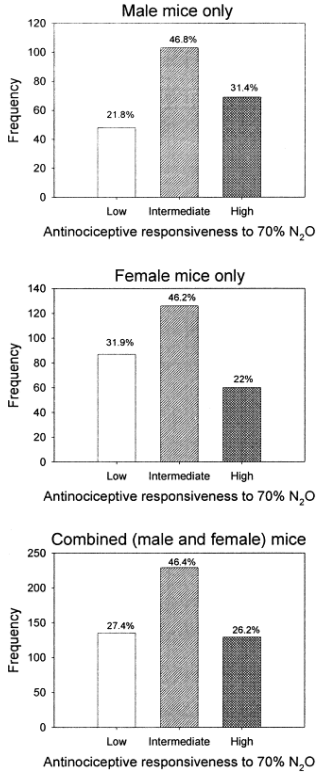

Fig. 1. Distribution of antinociceptive responsiveness of F_2 offspring to N_2O. The classification of responsiveness to N_2O antinociception was based on the following ranges of antinociceptive response to 70% N_2O: high, 90–100%;

intermediate, 11–89%; and low, <10% (including hyperalgesic responses or negative antinociceptive response). The number of animals screened was 220 male F_2 mice and 273 female F_2 mice for a grand total of 493 F_2 mice.

The MAINSCAN F_2 results are shown in Fig. 2. Of the 20 mouse chromosomes in our genome scan, only those 10 showing at least $P<0.01$ for a QTL or an interaction are shown. The most promising QTL were on proximal chromosome 2, distal chromosome 5 and distal chromosome 18, all showing LOD>2.0 for the F_2 data alone. These were very near the markers *D2Mit91*, *D5Mit409* and *D18Mit186* at 42, 89 and 45 cM, respectively. For the chromosomes 2 and 5 QTL, the B6 allele conferred higher antinociception scores than did the D2 allele, while the opposite was the case for the chromosome 18 QTL. This same pattern of results was also observed in our BXD study (Quock et al., 1996).

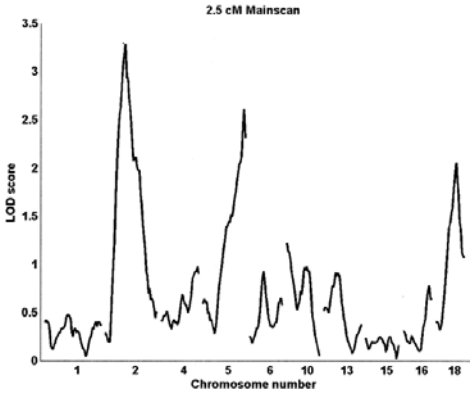

Fig. 2. LOD score plot (*df*=2) of F_2 QTL data at 2.5 cM resolution using the program MAINSCAN (Sen and Churchill, 2001). From the LOD scores presented, *P* values can be obtained from the expression $P=10^{-LOD}$. Only those chromosomes showing at least $P<0.01$ for a main effect in MAINSCAN or for an interaction in PAIRSCAN are shown.

The PAIRSCAN results are shown in Fig. 2 for the same 10 chromosomes shown in Fig. 2. This analysis found one significant interaction ($P=3\times10^{-5}$), that between a locus on chromosome 13 and another on chromosome 6, as shown by the arrow in Fig. 3. Fig. 4 shows this same interaction in terms of the antinociception scores for all nine genotypes possible between these two interacting loci. An interaction primarily of the dominance×dominance type is demonstrated by the fact that the heterozygotes at chromosome 13 show very high antinociception when the chromosome 6 genotype is homozygous B6 (shown as BB), but in marked contrast, the chromosome 13 heterozygotes show very low antinociception when the chromosome 6 genotype is either heterozygous or homozygous D2 (shown as Het or DD). Neither locus had a significant main effect in the MAINSCAN analysis, but emerged as significant only in the PAIRSCAN analysis due mainly to the strength of the interaction ($P=3\times10^{-5}$). The numbers of mice per group are shown within each bar of Fig. 4.

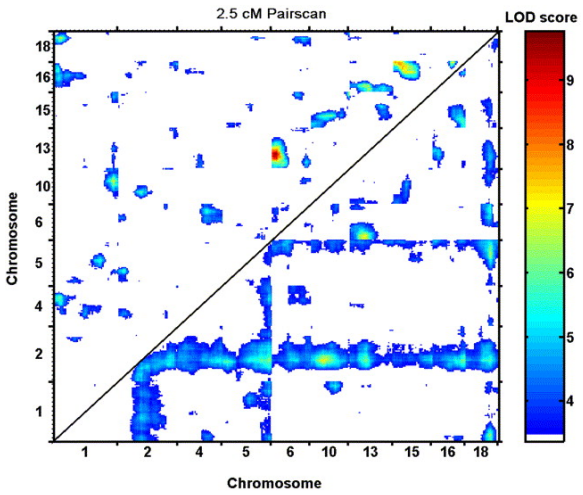

Fig. 3. Two-dimensional plot of PAIRSCAN results for the 10 chromosomes of greatest interest shown in Fig. 1. The lower half shows the LOD scores for the full model (*df*=8) for marker pairs, which includes the main effects of each marker plus their interaction, while the upper half shows LOD scores for the interaction alone (*df*=4). Areas in white failed to attain *P*<0.05 (<LOD 3.4 lower plot, <LOD 2.1 upper plot), while those exceeding this relaxed criterion are shaded from gray to black as a function of the LOD score scale shown at right. For visual clarity, the LOD scores in the upper half were increased by 1.64-fold. The one significant interaction ($P=3\times10^{-5}$) is denoted by the arrow. Several other interactions are shown, but our sample size did not have sufficient power to detect them as suggestive or significant (see Experimental Procedures).

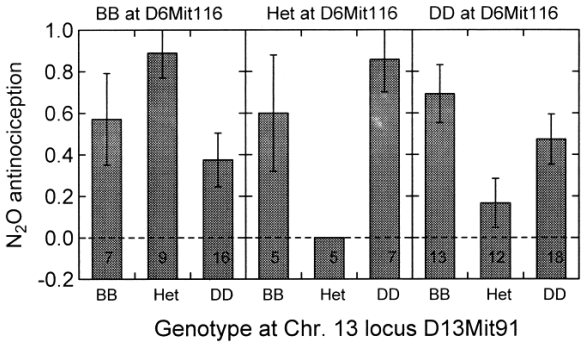

Fig. 4. The degree of N_2O antinociception (divided by 100) by genotype for the *D13Mit4* marker on chromosome 13 as a function of genotype at another locus (*D6Mit116*) on chromosome 6. The genotypes are symbolized BB, Het and DD, which stand for B6 homozygotes, heterozygotes and D2 homozygotes, respectively. An interaction ($P=3\times10^{-5}$) predominantly of the dominance×dominant type is demonstrated by the fact that the heterozygotes at chromosome 13 show very high antinociception when the chromosome 6 genotype is homozygous BB, but show very low antinociception when the chromosome 6 genotype is either heterozygous or homozygous DD. The numbers of mice per group are shown within each bar.

These MAINSCAN F_2 findings were combined with the earlier QTL analysis from BXD recombinant inbred mice (Quock et al., 1996). The combined results are shown in Table 1. The overall results when the *P*values from both populations were combined revealed two significant and one suggestive QTL. The significant QTL were on proximal chromosome 2 ($P=5\times10^{-5}$) and distal chromosome 5 ($P=1.3\times10^{-4}$), while the suggestive QTL was on distal chromosome 18 ($P=5\times10^{-4}$). These three were very near the markers *D2Mit91*, *D5Mit409* and *D18Mit186* at 42, 89 and 45 cM, respectively. However, the 95% confidence intervals for map location are in the range of about 20–35 cM for the two significant QTL (Darvasi and Soller, 1997), or about one-quarter to one-third the length of each chromosome. Table

1 also shows the proportion of the phenotypic (trait) variance expressed as a percent (%var) due to each of these three QTL that has been corrected for the upward bias that selective genotyping causes. The upward bias was estimated to be three-fold (Darvasi and Soller, 1992), thus the correction factor was 0.33. Because of this, the %var values given in Table 1 are estimates of what we would have observed had we genotyped all 493 mice in our study instead of only the top and bottom 10%.

Table 1. QTL that determine responsiveness to 70% N_2O for BXD and F_2data separately and for their combined *P* using Fisher's method for combining *P* values from independent experiments testing the same hypothesis[a]

BXD marker, F_2marker	Chromosome	BXD cM	BXD P	F_2cM	F_2 P	Combined P	F_2 %var, dom
D2Mit58, D2Mit91	2	51	0.007	44	0.0005	5.0×10^{-5}	5.1%, partial D2 dom
lapls2–5, D5Mit409	5	93	0.005	85	0.002	1.3×10^{-4}	3.7%, partial B6 dom
lapls3–12, D18Mit186	18	43	0.007	45	0.006	4.7×10^{-4}	3.1%, B6 overdom

[a] Only those QTL attaining either suggestive (chromosome 18) or significant status are shown based on the combined results (2nd column from right). For the F_2, data are for both genders combined.

When these three QTL were examined for gender differences, the chromosome 2 QTL showed a significant gender difference, with LOD 2.43 in males and LOD 0.39 in females (*P*=0.008 for the gender difference). This value for the chromosome 5 QTL was *P*=0.04 and for the chromosome 18 was *P*=0.06, both not significant. For all three, the QTL effect sizes (%var) were over two-fold larger in males than females, although this was significant only for chromosome 2.

Because our BXD study used only males, it is instructive to combine these results with only the male results from the F_2. The results were closely similar to those given when the F_2 data were based on both genders together (shown in Table 1) because all three QTL had larger effect sizes in males than females. The combined *P* for chromosomes 2, 5 and 18 were 2×10^{-4}, 7×10^{-5} and 5×10^{-4}, respectively, which compared well to the combined *P*s when both genders were combined shown in Table 1.

Discussion

Previous research assessing the responsiveness to N_2O antinociception in BXD recombinant inbred mouse strains resulted in the identification of eight markers distributed over seven chromosomes that were possibly associated with N_2O antinociception at *P*<0.01 (Quock et al., 1996). Based on a strong *P* value, accurate mapping of the marker, and a location in the middle of a sequence of markers with high correlation, the strongest correlations observed were the following: *Il2ra* on chromosome 2 (*P*<0.002); *Hbb* on chromosome 7 (*P*<0.002); *Hmg1rs7* on chromosome 16 (*P*<0.002); and *Gs15* on chromosome 19 (*P*<0.002). These QTL were suggestive rather than definitive because they fell well short of the *P*<0.0001 level needed for statistical significance for BXD QTL data (Belknap et al., 1996).

When the present F_2 QTL results were combined with the earlier QTL analysis from BXD recombinant inbred mice (Quock et al., 1996), the results revealed two significant and one suggestive QTL that influence responsiveness to N_2O antinociception on mid chromosome 2, distal chromosome 5 and mid chromosome 18. For the chromosomes 2 and 5 QTL, the B6 allele conferred higher antinociception scores than did the D2 allele in both BXD and F_2 populations, while the opposite was the case for chromosome 18 QTL.

This study is among the few to carry out a full genome search for epistasis, or the interaction between pairs of QTL in their influence on a trait of interest. To the usual additive and dominance variation as the

basis for detecting QTL in an F_2 (MAINSCAN), we add epistasis as a third source of genetic variation to facilitate detection (PAIRSCAN). The importance of an interaction lies in showing that both interacting loci have to be examined for their joint effects on the trait rather than each locus examined singly, and that interacting loci often are part of the same pathway or intertwined pathways, thus they can serve as clues to function at the pathway level. Unfortunately, the power to detect interactions between pairs of QTL is typically much smaller than that to detect single QTL in a given experiment. Nonetheless, a significant interaction did emerge in the PAIRSCAN study, that between a locus on chromosome 6 and another on chromosome 13. Thus, these two interacting QTL have now been detected as influencing antinociception even though neither had even a suggestive effect when examined singly (MAINSCAN).

Most QTL studies make use of a single large mapping population, most often an F_2 intercross between two inbred strains. This approach has a proven track record, and there is much to recommend it. However, in the present study we used two mapping populations for a number of reasons. The first is that replication of QTL results can be tested, and those that pass the test are more likely to represent robust QTL effects relatively insensitive to the influence of varying genetic background (inbred vs. segregating) and fluctuations in laboratory environment over time. Second, the specific strengths of each mapping population can be exploited to gain additional genetic information. For example, RI sets are very efficient in the detection of genetic correlations between two or more traits, while the F_2 is the least efficient for this purpose (Crabbe et al., 1990). Third, one of the populations (RI) is inbred strains providing stable and replicable genotypes. This allows the accumulation of genetic data across time and laboratories on the same genotypes or strains.

The QTL analysis used here is an example of a trait-driven analysis aimed ultimately at the discovery of new genes that are known from the outset to be trait relevant. Knowing the approximate map location of a QTL is often the all-important first step toward identifying the specific gene underlying a QTL. This is because of the large and increasing number of mapped genes of obvious neurochemical import in the mouse, making it likely that QTL map sites emerging from genome searches will immediately suggest plausible candidate genes previously mapped to the same region.

At this early stage in genetic dissection, the map resolution of each of our significant QTL is rather broad (20–35 cM), and consequently there are many genes within these intervals that may underlie our QTL. However, one candidate gene is worth noting—*Nos1* on chromosome 5 (65 cM), which encodes the neuronal form of the enzyme nitric oxide synthase (NOS) (wwww.informatics.jax.org) and maps near to our chromosome 5 QTL. This QTL has a peak LOD at 84 cM, but the confidence interval clearly encompasses the *Nos1* locus. The identification of this candidate gene is significant because in earlier studies, we implicated nitric oxide in N_2O antinociception. Pharmacological inhibition of NOS, particularly the neuronal isoform, antagonized the antinociceptive effect of N_2O in mice (McDonald et al., 1994, Ishikawa and Quock, 2003b). A recent study also reported that exposure to N_2O stimulated NOS enzyme activity in brains of N_2O-sensitive B6 mice but not N_2O-insensitive D2 mice (Ishikawa and Quock, 2003a). Though circumstantial, these results suggest that an insensitivity of the neuronal NOS enzyme in D2 mice to stimulation by N_2O may explain the insensitivity of the D2 strain to N_2O antinociception.

Further studies that can attain higher resolution mapping to 1 cM would narrow the number of possible genes down to less than 20—of which about half would likely be expressed in brain—and provide further evidence of the involvement of *Nos1* and other genes in N_2O antinociception.

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