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Increased mRNA Expression for the α_1 Subunit of the $GABA_A$ Receptor Following Nitrous Oxide Exposure in Mice

Lisa M. Johanek
Marquette University

William E. Cullinan
Marquette University, william.cullinan@marquette.edu

Linda K. Vaughn
Marquette University, linda.vaughn@marquette.edu

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Lisa M. Johanek
Department of Biomedical Sciences, Marquette University, PO Box 1881, Milwaukee WI 53201-1881, USA

William E. Cullinan
Department of Biomedical Sciences, Marquette University, PO Box 1881, Milwaukee WI 53201-1881, USA

Linda K. Vaughn
Department of Biomedical Sciences, Marquette University, PO Box 1881, Milwaukee WI 53201-1881, USA

Abstract

The mechanisms by which nitrous oxide (N_2O) produces physical dependence and withdrawal seizures are not well understood, but both N_2O and ethanol exert some of their effects via the $GABA_A$ receptor and several lines of evidence indicate that withdrawal from N_2O and ethanol may be produced through similar mechanisms. Expression levels of mRNA transcripts encoding several $GABA_A$ receptor subunits change with chronic ethanol exposure and, therefore, we hypothesized that N_2O exposure would produce changes in mRNA expression for the α_1 subunit. Male, Swiss–Webster mice, 10–12 weeks of age, were exposed for 48 h to either room air or a 75%:25% N_2O:O_2 environment. Brains were sectioned and mRNA for the α_1 subunit was detected by in situ

hybridization using an ^{35}S-labelled cRNA probe. N_2O exposure produced a significant increase in expression levels of the α_1 subunit mRNA in the cingulate cortex, the CA1/2 region of the hippocampus, the dentate gyrus, the subiculum, the medial septum, and the ventral tegmental area. These results lend support to the hypothesis that N_2O effects are produced, at least in part, through the $GABA_A$ receptor and that N_2O produces these effects through actions in the cingulate cortex, hippocampus, ventral tegmental area and medial septum. These results are also further evidence that ethanol and N_2O produce dependence and withdrawal through common mechanisms.

Neurotransmitters, modulators, transporters, and receptors, GABA receptors

Keywords

Nitrous oxide, $GABA_A$ receptor, $Alpha_1$ subunit, Mouse, In situ hybridization

1. Introduction

Nitrous oxide (N_2O) is an analgesic, anxiolytic, anesthetic drug with euphorogenic properties and abuse potential. Animals with prolonged exposure to N_2O experience physical dependence and withdrawal seizures [3], [18], [31], [36], [50]. Although it is widely believed that nitrous oxide interacts with central opioid mechanisms to produce its analgesic effect (for review see [48]) and through the $GABA_A$ receptor to produce its anxiolytic effect [13], [45], [49], the mechanisms by which N_2O produces physical dependence and withdrawal seizures remain unclear.

Several lines of evidence indicate that withdrawal from N_2O and ethanol may be produced through similar mechanisms. Withdrawal from N_2O and ethanol are both accompanied by withdrawal seizures [3], [18], [31], [36], [50], [53]. N_2O can suppress ethanol withdrawal seizures [3], ethanol can suppress N_2O withdrawal seizures [3], and cross-tolerance between N_2O and ethanol has been demonstrated [22]. In addition, sensitivity of various mouse strains to ethanol and N_2O correlate well [3], [21], and quantitative trait loci analysis of BXD recombinant inbred mouse strains demonstrates a genetic correlation between N_2O and ethanol withdrawal [4].

Some of the effects of both N_2O and ethanol, including anxiolytic effects and withdrawal symptoms, are known to occur through interaction with the $GABA_A$ receptor. The anxiolytic effects of N_2O are sensitive to antagonism by the benzodiazepine antagonist flumazenil and the anxiolytic effects are significantly reduced in benzodiazepine-tolerant animals [13], [45]. There is also evidence that withdrawal responses following exposure to N_2O involve the $GABA_A$ receptor. For example, injection of the benzodiazepine partial inverse agonist Ro 15-4513 significantly increased the frequency of withdrawal seizures in N_2O-exposed mice [56], an effect also seen with ethanol-induced withdrawal seizures [2], [27].

$GABA_A$ receptors are heterooligomeric complexes consisting of numerous isoforms of several classes of subunits (α, β, γ, δ, ρ). Since recombinant expression studies have shown that the pharmacology and the binding characteristics of $GABA_A$ receptor are altered by the expression of different subunit combinations [1], [7], [10], [25], [28], [37], [38], [44], [51], [52], it has been hypothesized that one mechanism by which ethanol alters $GABA_A$ receptor function is by differentially affecting the expression of $GABA_A$ receptor subunits and thus altering the composition of $GABA_A$ receptors and their responsiveness to GABA [34], [39], [41]. Expression levels of mRNA transcripts encoding several $GABA_A$ receptor subunits are altered by chronic ethanol exposure, with consistent findings of decreased mRNA expression for the α_1 subunit in rat cortex following chronic ethanol exposure [8], [12], [32], [33], [34], [39], [41]. A number of studies have detected changes in other subunits of the $GABA_A$ receptor in either rat cortex and cerebellum following chronic ethanol, including changes in α_{2-6}, [8], [11], [12], [33], [34], [39], β_{1-3}[11], [35], and γ_1[11], [12], with others reporting no differences in expression of α_3[34], [39], α_5[12] or β_{1-3}[12], [40]. However, relatively few studies have examined brain regions other than cortex or cerebellum. These studies have found that durations longer than the typical 2 weeks of ethanol exposures are

necessary to produce changes in $GABA_A$ receptor subunit composition. Forty days of chronic ethanol consumption were necessary to produce an increase in the α_4 subunit peptide in hippocampus [32]. Gene expression for the hippocampal α_1, α_2, α_3, $\beta_{2/3}$ or γ_2 subunits was unchanged at that time. Others found that a 12-week, but not 4-week, ethanol exposure induced changes in α_1 and α_5 subunit expression in the hippocampus and α_1 subunit levels in the ventral tegmental area [8], [42]. No changes were found in nucleus accumbens or substantia nigra [8].

Results of ethanol exposure on $GABA_A$ receptor subunit composition in mouse brain have been mixed, with results largely dependent upon mouse strain and brain region. For instance, following ethanol treatment, expression of whole brain α_1 mRNA was found to be decreased in Withdrawal Seizure Prone (WSP) and unchanged in Withdrawal Seizure Resistant (WSR) mice [6] but increased in ddy mice [19]. On the other hand, α_1 mRNA expression was reportedly increased in the cerebellum but decreased in the whole brains of C57BL/6J (C6) mice [60]. Levels of α_6 mRNA were decreased in WSR mice, unchanged in WSP mice [6], and increased in C6 mice [60]. Levels of γ_2 mRNA were increased by ethanol in C6 mice [60] but not WSP or WSR mice [6].

Because both N_2O and ethanol exert some of their effects via the $GABA_A$ receptor, and evidence indicates that ethanol and N_2O produce dependence and withdrawal through similar mechanisms, we investigated the effect of N_2O exposure on mRNA expression for the α_1 subunit of the $GABA_A$ receptor.

Further, we felt it important to conduct an extensive neuroanatomical study, both because multiple brain regions are thought to be important in the development of drug dependency and withdrawal responses, and because of the wide-ranging effects of N_2O on the brain. Previous work has shown that withdrawal seizures can be evoked following durations of N_2O exposures ranging from 30 min to 48 h (62). For this neuroanatomical study of the distribution of N_2O-induced changes in mRNA expression, a 48-h duration was chosen to maximize opportunity for alterations mRNA expression levels.

2. Materials and methods

2.1. Nitrous oxide exposure

Male, Swiss–Webster mice (Charles River, Portage, MI), 10–12 weeks of age, were exposed for 48 h to either room air or a 75%:25% N_2O:O_2 environment. Three cages of five mice were placed in a modified infant incubator [26] with a N_2O delivery rate of 6 liter/min and an O_2 delivery rate of 1.5 liter/min. Gases were delivered using a standard nitrous oxide/oxygen anesthesia machine (Adec, Newburg, Oregon). The exhausted gas was vented to a nearby fumehood. Mice were removed from the N_2O, left in room air for 10 minutes and sacrificed by rapid decapitation. Whole brains were immediately removed, frozen in isopentane at −40°C for 30 s, wrapped in parafilm, and stored at −70°C

2.2. In situ hybridization

2.2.1. Tissue fixation

Serial cryostat sections of brain (14 μm) were mounted on polylysine-coated slides and stored at −70°C until use. Slices were fixed in 4% buffered paraformaldehyde for 2 h and washed four times with 2×SSC (saline–sodium citrate buffer) (pH=7.4). Tissue sections were treated with proteinase K (0.1 μg/ml) for 8 min at 37°C, washed with distilled water and rinsed with 2×SSC. Slides were subsequently acetylated with 0.25% acetic anhydride in a 0.1 M triethanolamine buffer (pH=8.0) for 15 minutes. Finally, slides were washed in 2×SSC three times and dehydrated in a successive series of alcohols.

2.2.2. Probe labeling

A riboprobe complementary to mRNA transcript encoding the α_1 subunit of the $GABA_A$ receptor was synthesized according to standard in vitro transcription methodology. Briefly, a cRNA probe was generated in a reaction

(total volume=10 µl) containing 125 µCi ^{35}S UTP (Amersham, 20 µCi/ml), 1 µl each of 10 mM stocks of ATP, CTP, and GTP, 2 µl 5×transcription buffer, 1 µl 0.1 M dithiothreitol, 1 µl linearized plasmid DNA (1 µg/µl), 1 µl distilled water, 0.5 µl of RNase inhibitor (40 U/µl), and 1.5 µl T7 polymerase. The reaction was allowed to proceed at 37°C for 2 h followed by separation of labeled probe from reaction contents on a G50-sephadex column. Probes were diluted in hybridization buffer (Amresco, Solon, OH) consisting of 45% formamide, 10% dextran sulfate, 3×SSC, 50 mM sodium phosphate buffer (pH=7.4), 1×Denhardt's solution, 0.1 mg/ml sheared salmon sperm DNA, and 0.1 mg/ml yeast, to yield approximately 1 500 000 cpm/35 µl buffer. The $GABA_A$-receptor cDNA was kindly provided by Dr. A. Tobin (UCLA) and was subcloned into PGem3Z and linearized with Hind III to transcribe a cRNA probe of 842 nts.

2.2.3. Hybridization histochemistry

Tissue sections were apposed to coverslips containing 35 µl probe and stored for 15 h at 55°C in sealed chambers moistened with a 45% formamide solution. Following hybridization, coverslips were removed in 2×SSC, the slides were washed extensively in 2×SSC and then treated with RNase A (200 µg/ml) in Tris buffer for 90 minutes at 37°C. This was followed by several rinses in decreasing concentrations of SSC (2×SSC, 1×SSC, 0.5×SSC, and 0.2×SSC), and washing at 65°C for 1 h in 0.2×SSC. Slides were then washed in 0.2×SSC at room temperature and dehydrated through graded alcohol concentrations. Slides were exposed to Kodak BioMax X-ray film for 16 h and developed in GBX developer.

2.2.4. Imaging

X-ray images were digitized and analyzed using NIH Image software. Identification of brain regions was determined using the atlas of Franklin and Paxinos [14]. Depending on the size of the brain region of interest, between 3 and 80 gray value signals for each specific brain region were obtained per mouse (*n*=5 control mice, *n*=8 N_2O exposed mice). Mean gray values were determined for each brain region per mouse after subtraction of background values determined from adjacent brain regions which lacked specific expression of the $GABA_A$ receptor.

2.3. Statistics

Two-way analysis of variance was performed to test whether there were differences in mean gray values in 20 brain regions between N_2O and room air exposed mice. To correct for heteroscedasticity, the data was transformed using the equation

[62]. Post-hoc analysis to determine which brain regions accounted for the statistically significant main effect seen in treatment groups was performed using the Tukey multiple comparison test.

3. Results

The regional expression pattern of the α_1 subunit mRNA was similar to that previously found in rat [29], [30], [43], [57] and long-sleep and short-sleep mouse brain [61]. Widespread distribution was noted, with highest expression densities in the cerebellar Purkinje layer and the ventral tegmental nucleus, high levels in the cortical primary and secondary somatosensory cortex, dense layer of the piriform cortex, CA1/2 region of the hippocampus and subiculum, granular layer of the cerebellum, red nucleus and the medial septum. Moderate to high levels were found in the cingulate and motor cortex, CA3 region of the hippocampus, dentate gyrus, molecular layer of the cerebellum, and globus pallidus. Low to moderate levels were found in the basolateral, medial and central amygdaloid nucleus, and ventral tegmental area (VTA) (Fig. 1). The regional distribution of the $GABA_A$ receptor α_1 subunit mRNA in N_2O and room air exposed mice is shown in Fig. 2, Fig. 3.

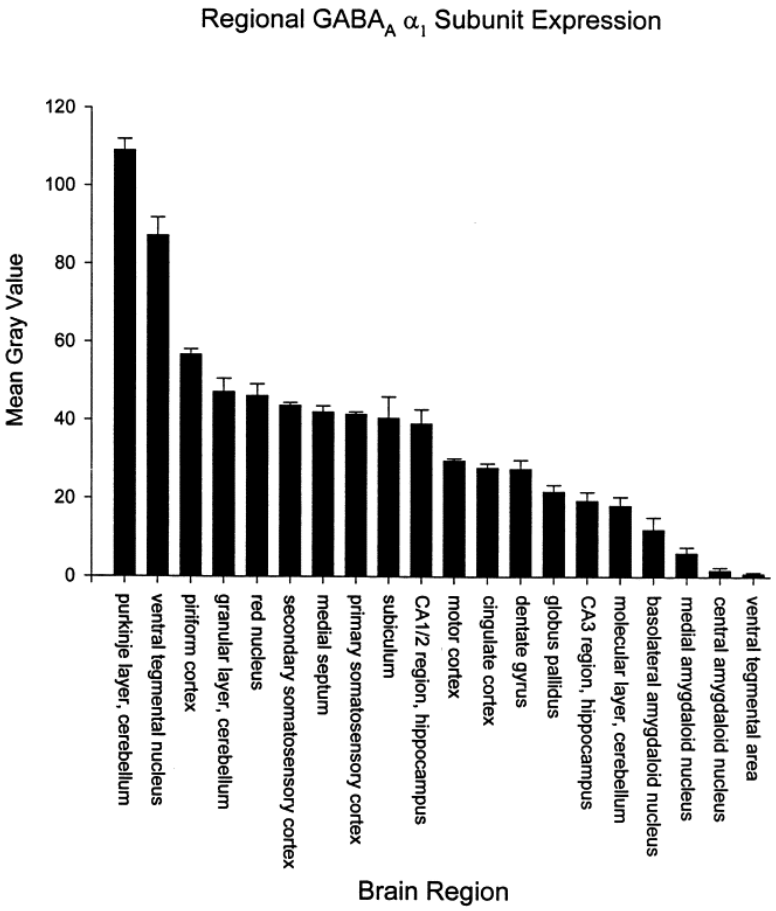

Fig. 1. Regional distribution of expression of GABA$_A$ α_1 subunit mRNA in control mice. Mean gray values, determined from multiple sections for each specific brain region, were determined for each mouse. The average mean gray values for 5 control mice±S.E.M. are plotted.

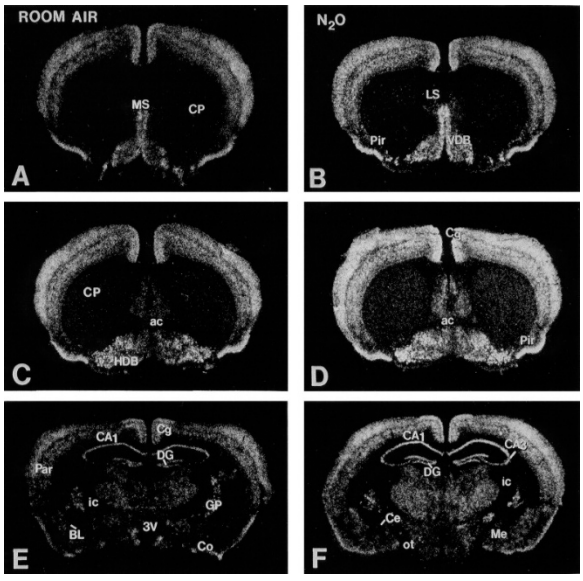

Fig. 2. Darkfield photomicrographs of coronal sections at three forebrain levels illustrate the brain distribution of mRNA transcripts encoding the α_1 subunit of the GABA$_A$ receptor in mice exposed to room air (A–E) or 75% N_2O (B–F) for 48 h. Abbreviations: ac, anterior commissure; BL, basolateral amygdaloid nucleus; CA1, CA3, hippocampal subfields; CE, central amygdaloid nucleus; Cg, cingulate gyrus; Co, cortical amygdaloid nucleus; CP,

caudate putamen; DG, dentate gyrus; GP, globus pallidus; HDB, horizontal limb of the diagonal band nucleus; ic, internal capsule, LS, lateral septal nucleus; Me, medial amygdaloid nucleus; MS, medial septal nucleus; Par, parietal cortex, Pir, piriform cortex; VDB, vertical limb of the diagonal band nucleus, 3V, third ventricle.

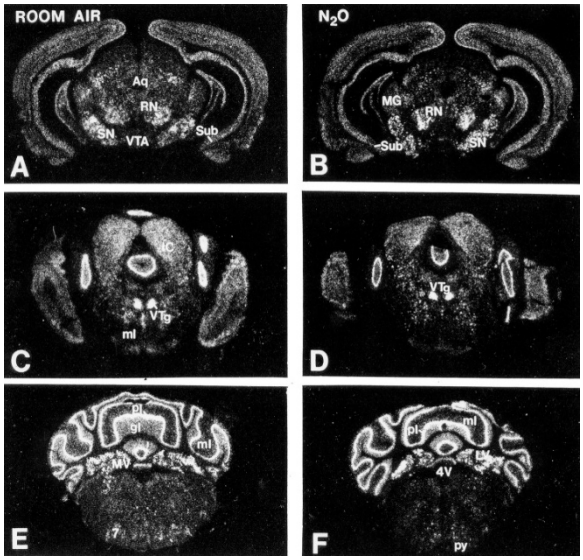

Fig. 3. Darkfield photomicrographs of coronal sections at three brainstem levels illustrate the brain distribution of mRNA transcripts encoding the α_1 subunit of the $GABA_A$ receptor in mice exposed to room air (A, C, E) or 75% N_2O (B, D, F) for 48 h. Abbreviations: Aq, cerebral aqueduct; gl, granular layer, cerebellum; IC, inferior colliculus; LV, lateral vestibular nucleus; MG, medial geniculate nucleus; ml, molecular layer, cerebellum; MV, medial vestibular nucleus; pl, purkinje layer, cerebellum; py, pyramidal tract; RN, red nucleus; SN, substantia nigra; Sub, subiculum; VTA, ventral tegmental area; Vtg, ventral tegmental nucleus; 4V, fourth ventricle; 7, facial nucleus.

Exposure of mice to 48 h of 75% N_2O produced a statistically significant increase in expression levels of mRNA for the α_1 subunit of the $GABA_A$ receptor (P<0.001, two-way ANOVA). There was also a statistically significant difference in expression levels of mRNA between brain regions (P<0.001) and a statistically significant interaction between treatment and brain region (P□0.023). Post-hoc statistical analysis using the Tukey test found significant increases following N_2O exposure in the cingulate cortex (P□0.002), the CA1/2 region of the hippocampus (P□0.005), the dentate gyrus (P□0.042), the subiculum (P□0.047), the medial septum (P□0.004), and the ventral tegmental area (P□0.034) (Table 1).

Table 1. The effect of nitrous oxide exposure on $GABA_A$ α_1 subunit mRNA expression[a]

Brain region	Room air exposure	N_2O exposure	% change	Significance
Cingulate cortex	27.90±1.09	38.03±1.68	36.3	P=0.002**
Motor cortex	29.66±0.60	35.44±0.90	19.5	P=0.082
Primary somatosensory cortex	41.46±0.63	46.92±1.10	13.2	P=0.167
Secondary somatosensory cortex	43.73±0.71	44.50±1.27	1.8	P=0.851
Piriform cortex	56.80±1.38	61.51±2.67	8.3	P=0.308
CA1/2 region, Hippocampus	39.11±3.63	49.85±2.74	27.5	P=0.005**
CA3 region, Hippocampus	19.44±2.22	25.09±1.85	29.1	P=0.214
Dentate gyrus	27.58±2.28	34.08±1.78	23.6	P=0.042*
Subiculum	40.50±5.46	47.76±3.04	17.9	P=0.047*
Medial septum	42.04±1.55	53.77±2.57	27.9	P=0.004**
Ventral tegmental area	0.76±0.40	2.43±0.47	219.7	P=0.034*
Purkinje layer, cerebellum	109.00±2.96	116.74±3.76	7.1	P=0.228

Granular layer, cerebellum	47.20±3.42	49.55±3.82	5.0	*P*=0.618
Molecular layer, cerebellum	18.23±2.21	17.23±1.98	−5.5	*P*=0.672
Basolateral amygdaloid nucleus	12.08±3.11	12.31±0.78	1.9	*P*=0.682
Medial amygdaloid nucleus	6.07±1.51	7.39±0.91	21.7	*P*=0.279
Central amygdaloid nucleus	1.62±0.79	0.50±0.35	−69.1	*P*=0.050
Red nucleus	46.19±2.99	42.10±3.23	−8.9	*P*=0.278
Ventral tegmental nucleus	87.20±4.6	83.58±5.06	−4.2	*P*=0.484
Globus pallidus	21.75±1.67	22.91±1.16	5.3	*P*=0.666

a. Levels of expression were measured using computerized image analysis and mean gray values were calculated from multiple sections throughout each region for each mouse. Data are reported as the average mean gray values±S.E.M. for mice exposed to room air (*n*=5) or nitrous oxide (*n*=8).

*Statistical significance was determined by the Tukey multiple comparison test following two-way analysis of variance. **P*<0.05; ***P*≤0.005.

4. Discussion

The present studies are the first to identify specific brain regions affected by N_2O exposure and indicate an up regulation of $GABA_A$ α_1 subunit mRNA in the cingulate cortex, the hippocampus, the medial septum, and the VTA. Interestingly, these brain regions have all been implicated as sites of action of ethanol, and ethanol-induced changes in $GABA_A$ receptor subunit expression have been identified in the cortex, hippocampus and VTA. Further, the VTA, as part of the mesolimbic dopaminergic pathway, has long been thought to be involved in the reinforcing actions of drugs of abuse, including ethanol [23], [24], [58], [59]. Ethanol can stimulate the activity of VTA dopaminergic neurons, and has been found to be reinforcing when administered directly into the VTA in operant conditioning experiments [15]. Mesolimbic GABAergic systems are also thought to be involved in the ethanol reinforcement properties, since muscimol injected into the VTA was shown to alter ethanol-reinforced responding [20].

Actions of ethanol and N_2O on the GABAergic system of the medial septum may be of particular functional importance. While the effects of ethanol on $GABA_A$ receptor subunit composition in the medial septum have not been investigated, other effects of ethanol on this brain region have been extensively examined. The medial septum has been implicated as a brain region that influences the ethanol-induced impairment of nonspatial working memory [17] and depression of locomotion [5], as well as the sedative action of ethanol, enhancing the GABA-mediated inhibition of medial septal neuronal activity [16], [54]. Chronic ethanol exposure also produces long term effects on the septal GABAergic system, decreasing GABA's ability to inhibit ethanol-sensitive cells in the medial septum after ethanol withdrawal [9]. Thus, the changes in $GABA_A$ α_1 subunit expression observed in the present study both support the hypothesis that N_2O effects are mediated, at least in part, through $GABA_A$ receptors and provide further evidence for similar mechanisms of action of N_2O and ethanol.

Importantly, the increases in $GABA_A$ α_1 subunit expression detected in the present study were not generalized to all regions under investigation. Areas examined in which differences could not be detected included the cerebellum, globus pallidus, red nucleus, basolateral and medial amygdaloid nuclei, as well as cortical areas such as the somatosensory and the piriform cortices. Interestingly, within the hippocampal formation, an up-regulation of α_1 subunits was noted within the dentate gyrus, CA1 and subiculum, but not in CA3, further suggesting the site-specific nature of the experimental effect. Moreover, regions in which increases were detected have all been implicated in aspects of ethanol addiction/withdrawal. If the present mRNA changes are indeed paralleled by similar increases at the protein level, the findings provide further evidence for a prominent role for GABAergic mechanisms in the reinforcing properties of N_2O and ethanol, and may yield insights into the sequence of cellular events underlying these processes.

The increase in α_1 subunit mRNA levels seen following N_2O exposure may reflect a generalized increase in the concentration of $GABA_A$ receptors. It is also possible that N_2O exposure causes a modification of receptor composition, with an increase in the proportion of $GABA_A$ receptors containing the α_1 subunit. There is evidence that ethanol can produce such an effect in rat cortex, where it has been shown that exposure to ethanol results in a decrease in α_1 subunit mRNA and protein approximately equal to the increase in the α_4 subunit mRNA and protein [11], [12]. A similar effect was found in the hippocampus, where long term administration of ethanol produced a decrease α_1 subunit mRNA and an increase in α_5 mRNA levels [8]. Such a change in subunit composition, with or without a change in the overall number of $GABA_A$ receptors, could significantly change $GABA_A$ receptor function, since the pharmacology and the binding characteristics of $GABA_A$ receptor have been shown to be influenced by expression of different $GABA_A$ receptor subunit combinations [1], [7], [10], [25], [28], [37], [38], [44], [51], [52]. Moreover, an increase in mRNA expression for the α_1 subunit produced by ethanol exposure in mice was previously shown to correspond to a decrease in GABA-dependent Cl^- flux [19]. The functional impact of N_2O-induced increases in the α_1 mRNA expression might similarly alter the efficacy of GABAergic inhibition. It should be noted, however, that N_2O-induced changes in mRNA levels for α_1 subunit may not correlate with changes in protein and/or changes in $GABA_A$ receptor stoichiometry.

The functional significance of the changes in $GABA_A$ receptor subunit expression produced by N_2O remains to be determined. Mouse strain has been shown to be an important variable in responsiveness to both the analgesic effects of N_2O [46], [47] and the ability of N_2O to induce withdrawal seizures [55]. Moreover, ethanol studies have shown that mouse strain can influence the effect of ethanol on $GABA_A$ receptor subunit expression [6], [19], [60]. Therefore, determining the effect of N_2O on $GABA_A$ receptor subunit expression in other mouse strains is an important consideration in the analysis of the functional impact of N_2O-induced changes in the $GABA_A$ receptor. In addition, results of previous ethanol studies have shown that effects on $GABA_A$ receptor subunit expression vary with the duration of drug exposure. Expression of α_1, α_6, β_3 and γ_2 subunits were differentially affected by acute and chronic ethanol administration [60] and varying durations of chronic ethanol exposures were necessary for changes in $GABA_A$ receptor subunit expression in different brain regions [8], [42]. Varying the duration of N_2O exposure may also provide insight into the functional impact of N_2O-induced changes in the $GABA_A$ receptor.

The N_2O-induced increase in $GABA_A$ receptor α_1 subunit mRNA expression found in the present experiments supports the hypothesis that N_2O effects are produced, at least in part, through the $GABA_A$ receptor, and localize the N_2O-induced effects to the cingulate cortex, hippocampus, ventral tegmental area and medial septum. These results are also consistent with the hypothesis that ethanol and N_2O produce dependence and withdrawal through common mechanisms. Future studies on the effect of N_2O on the full complement of subunits of the $GABA_A$ receptor, as well as additional receptor types, will be required for a more complete understanding of the neuronal mechanism of action of N_2O.

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