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Effect of Nitrous Oxide on the Concentrations of Opioid Peptides, Substance P, and LHRH in the Brain and β-Endorphin in the Pituitary

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Many studies have indicated that nitrous oxide (N_2O) exposure results in specific effects on the reproductive system, some of which are antigonadotropic. The neurochemical events regulating the pituitary–gonadal axis are probably influenced by N_2O, but precise documentation is lacking. The effects of exposure to 30% N_2O in air on the brain tissue concentrations of luteinizing hormone releasing hormone (LHRH), substance P (SP), met-enkephalin, and β-endorphin and on β-endorphin concentrations of the pituitary gland are described in this study. Female rats were exposed to either N_2O or air for 8 hr a day over one estrous cycle, and the brain and pituitary tissues were collected and processed. Neuropeptide concentrations were measured by specific radioimmunoassays. Exposure to N_2O resulted in significant elevation of LHRH in the preoptic area, with a concomitant decrease in SP. The SP concentration of the medial basal hypothalamus was significantly elevated in N_2O-exposed animals. Exposure to N_2O resulted in significant increases in met-enkephalin in the brainstem area and β-endorphin in the pituitary. These results suggest that exposure to N_2O alters the interactive neural system activity regulating gonadotropin secretion from the pituitary. The significance of increased met-enkephalin in the brainstem of N_2O-exposed animals is not known.

Since its first use in 1844, nitrous oxide (N_2O) has been regarded as a safe anesthetic. However, studies over the past 35 years have demonstrated that chronic exposure to high concentrations of N_2O results in leukopenia and embryo toxicity.[1,2] In recent years it has been suggested that operating room and dental staff exposed occupationally to N_2O exhibit increased incidence of reproductive and health problems.[3] Although the hazards associated with occupational exposure to N_2O in the operating room were documented as early as 1974,[4] the extent of risk to dental staff is unknown. Chronic exposure to low levels of N_2O has been implicated in infertility problems in male[5] and female[6] rats. Adverse effects of N_2O include decreased size of the litter and litter weight,[7] and an increased risk of late pregnancy termination.[4] Female Wistar rats exposed to 60% N_2O in oxygen for 5 hr/day over 15 days exhibited absence of ovulation and progressive reduction and eventual disappearance of the proestrous phase, resulting in an absolute functional incapacity for procreation.[8]

Previous studies performed in our laboratory[9] indicated that 30% N_2O (at 1.6 L/min, 8 hr/day for 4 days) results in (1) a threefold increase in luteinizing hormone releasing hormone (LHRH) immunopositive cells in the hypothalamus of rats sacrificed on the morning of proestrous; (2) a 50% decrease in fertility rate; and (3) disrupted estrous cycles arrested in constant proestrous, lasting up to 3 wk. These results in general indicate that a subchronic exposure to N_2O results in a disruption of the hypothalamo–pituitary–gonadal axis. Furthermore, our immunocytochemical data on the LHRH neurons indicate that N_2O probably has a significant effect on the central neural components regulating the hypophyseal–gonadal axis. Not much is known at present about the mechanism of action of N_2O as an anesthetic agent or about how it influences the hypothalamic component of reproductive regulation.

Endogenous opioid neurons represent one of the important controlling systems for the release of LHRH within

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the diencephalon.[10,11] The popular model for opioid regulation of reproduction suggests that inhibition/disinhibition of opioid control of LHRH release is selectively regulated by internal and external factors. We also have demonstrated previously that agents that inhibit the hypothalamic component of reproductive regulation selectively activate opioid neurons.[12–14] Others also have demonstrated that gonadotropin suppression may involve the endogenous opioid system.[10,11] The undecapeptide substance P (SP) also appears to play a regulatory role on the release of gonadotropins either directly or through the regulation of LHRH.[15–17] In addition, SP is implicated in the regulation of pain pathways[18] and has extensive interactions with the endogenous opioid system.[19] This study was therefore designed to evaluate the effects of N_2O on, not only the LHRH content of the diencephalon, but also the concentrations of SP, β-endorphin, and met-enkephalin in the brain and β-endorphin in the pituitary gland. Extrahypothalamic areas of the brain were included in order to obtain preliminary information regarding SP and opioid peptides in these interactive neural systems.

METHODS

Adult virgin Sprague–Dawley rats (Charles River, Wilmington, MA) weighing 180–200 g were housed in polypropylene animal cages, four per cage, in a temperature-controlled room (21° ± 1° C). The animals were maintained under a 12-hr light cycle (lights on 0600–1800 hr); food and water were made available freely. Following shipment, all animals were allowed to adjust to the room environment for 2 wk before placement in an environmental chamber.[9] The chamber was approximately a 2-ft cube made out of clear acrylic plastic, with a hinged door that could be closed air-tight. The inlet and outlet ports of the chamber were appropriately connected to gas lines and evacuation lines. Once placed in the chamber, rats were acclimated for 1 week, at which time vaginal smears were initiated for all rats. A record of daily vaginal cytology was maintained for all rats. Those animals exhibiting two, consecutive 4-day estrous cycles were selected for either the experimental (n = 8) or control group (n = 8) by a random process.

The experimental rats were exposed to a 30% N_2O/70% air mixture delivered at a flow rate of 1.6 L/min for 8 hr a day for 4 days (one ovulatory cycle) in the environmental chamber. The concentration of N_2O was monitored throughout the study period by random gas sampling using a MIRAN 101 specific vapor analyzer (Foxboro Corp., South Norwalk, CT). The control animals were housed in an identical environmental chamber but received compressed air at a similar flow rate and duration as the experimental animals. At the termination of exposure to either N_2O/air or air alone, rats were sacrificed by decapitation. The brain and entire pituitary gland from each animal were rapidly removed and kept on ice. The neural lobe of the pituitary was removed by forceps and discarded, and the intermediate–anterior lobe of the pituitary was frozen immediately on dry ice. Because of a cleft, the neural lobe can easily be peeled off from the rest of the pituitary.[20] The brain was further dissected grossly into the preoptic area (POA), medial basal hypothalamus (MBH), pons, and medulla as described before.[20] The entire dissection and isolation of individual tissue pieces were usually achieved within 5 min after decapitation.

Extraction of Neuropeptides from Tissues

Tissues were individually homogenized by sonic disruption in 2 mL of 2N acetic acid. After centrifugation (10,000 RPM, 10 min), the supernatant was saved, and the step repeated. The pooled supernatants were lyophilized and reextracted into the aqueous phase of 50% acetonitrile with 0.1% trifluoroacetic acid (TFA) and 50% chloroform as described before.[14] The aqueous phase was lyophilized in two separate aliquots (one for met-enkephalin and the second for other peptides) and reconstituted either in distilled water (met-enkephalin assay) or RIA buffer[20] and used for peptide radioimmunoassays.

Radioimmunoassays

The LHRH radioimmunoassay procedure has been described previously,[21] and the anti-LHRH specificity was characterized by immunohistochemical studies.[22] We used anti-LHRH #100 at a final dilution of 1:150,000. At this dilution, the antiserum binds about 40% of the ^{125}I–LHRH. We have recently developed a radioimmunoassay for the methionine sulfoxide derivative of met-enkephalin, similar to the assay described by Clemente-Jones et al.[23] Methionine-enkephalin oxidized by chloramine-T treatment was purified by high-pressure liquid chromatography and subsequently conjugated to bovine serum albumin by glutaraldehyde. The anti-met-enkephalin sulfoxide #25 is highly sensitive and very specific for oxidized met-enkephalin. The cross-reactivity of the antiserum #25 has been described before.[14] The β-endorphin radioimmunoassay also was developed and characterized by our laboratory previously.[14] The SP radioimmunoassay was developed by Dr. Kream (Tufts School of Medicine), and the assay has been extensively validated.[10,21,24]

Statistical Analysis

The data were analyzed using the Stat-View 512 program (Brain Power Inc., Calabasas, CA) adapted for the Macintosh SE. Differences in neuropeptide concentrations were

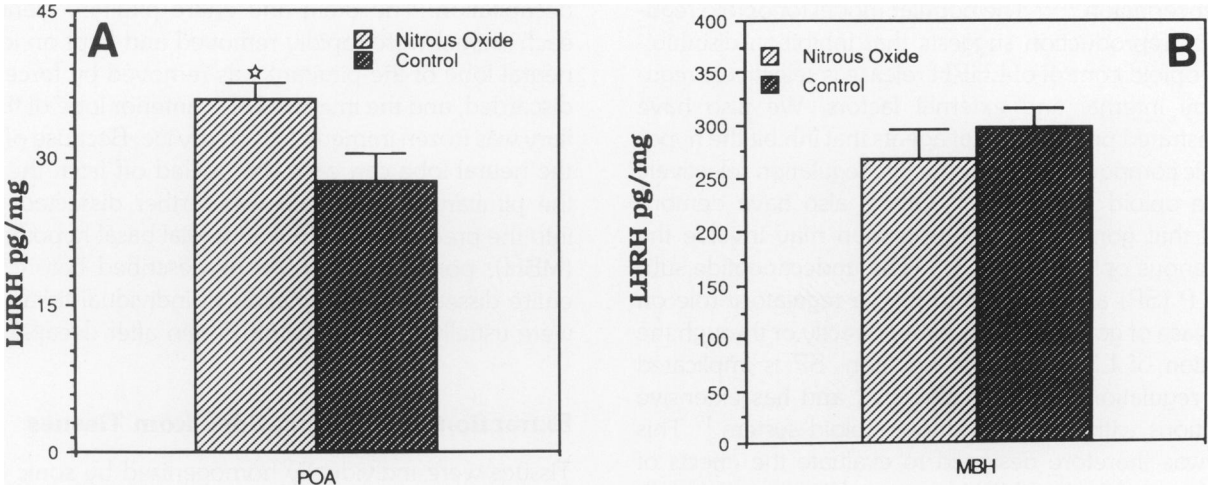


Figure 1. Tissue concentrations of LHRH in **(A)** the preoptic area (POA) and **(B)** medial basal hypothalamus (MBH) of rats exposed to either 30% N_2O or air (control). Open star indicates statistical significance at $P < 0.05$; brackets indicate the standard error.

analyzed using a Student's *t*-test, and differences were considered significant at $P < 0.05$.

RESULTS

Seven of the eight animals exposed to N_2O were found arrested in the proestrous stage of the ovulatory cycle. Corresponding smears for the control group indicated the presence of normal estrous cycles.

The animals exposed to N_2O demonstrated a substantial increase in LHRH in the POA when compared with the control animals (Figure 1*A*). However, LHRH in the MBH of the N_2O-exposed animals was not significantly different from that of control animals (Figure 1*B*).

Exposure to N_2O resulted in significant elevations of met-enkephalin in both the pons and medulla (Figure 2). However, N_2O exposure did not change concentrations of met-enkephalin in either the POA or MBH.

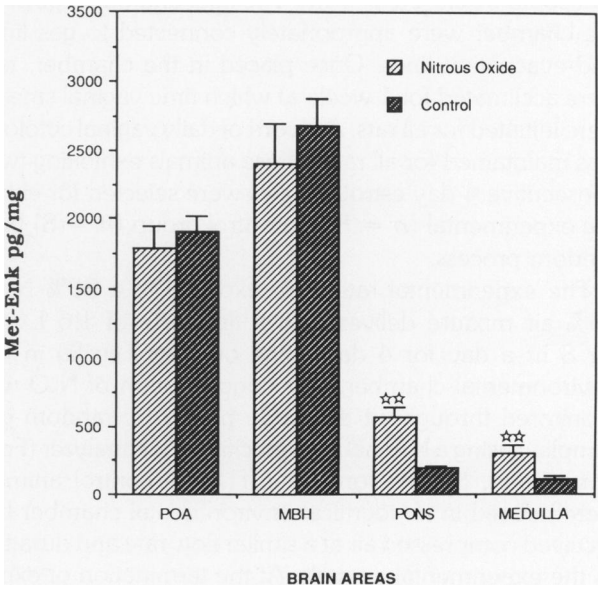
N_2O-exposed animals did not significantly differ with regard to β-endorphin in either the POA or MBH when compared with the control animals (Figure 3). However, the concentration of β-endorphin in the intermediate–anterior lobes of the pituitary was significantly elevated (Figure 4).

The concentration of SP in the POA of N_2O-exposed animals was significantly decreased in comparison with control animals (Figure 5). In contrast, SP in the MBH of N_2O-exposed animals was significantly elevated. Exposure to N_2O did not result in significant changes in SP in either the pons or medulla.

DISCUSSION

In the present study, we used 30% N_2O mixed with air instead of oxygen. Although the N_2O-exposed rats were in effect getting 30% less oxygen than the control animals, their behavior was not altered by the mild hypoxia. The corticosterone concentrations of N_2O-exposed animals were not different from control animals, indicating that the animals were not overtly stressed by the mild hypoxic

Figure 2. Met-enkephalin concentrations in the POA, MBH, pons, and medulla of rats exposed to either 30% N_2O or air. The levels of met-enkephalin in the pons and medulla were significantly higher (two stars, $P < 0.01$) in N_2O-exposed animals.



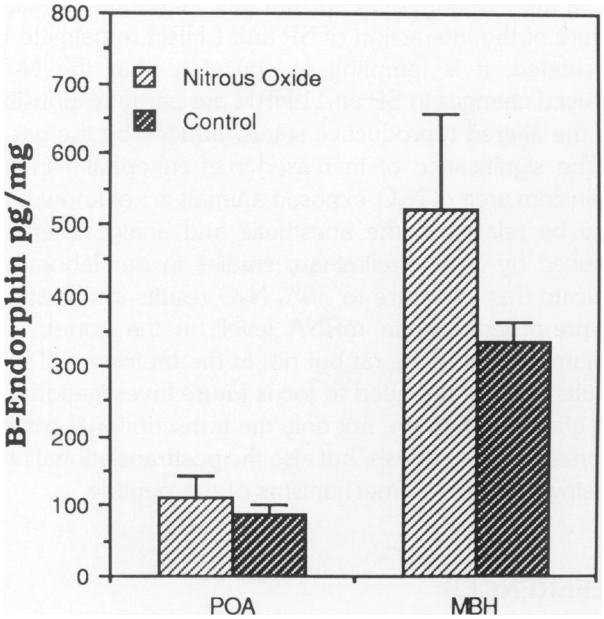


Figure 3. β-Endorphin content of the POA and MBH from rats exposed to either 30% N_2O or air.

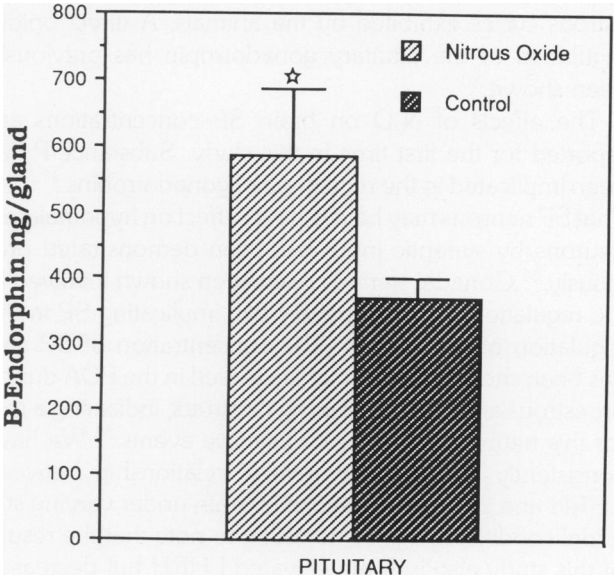
condition [N_2O animals, 63.5 ± 7.7 μg/dL; controls, 67.1 ± 4.9 μg/dL, (mean ± standard error)]. In addition, there is no evidence to indicate that short-term exposure to mildly hypoxic conditions results in antigonadotropic effects.

Results of this study indicate that exposure to 30% N_2O for 8 hr a day for 4 days brings about significant neurochemical changes in the animals. Our previous study[9] indicated that N_2O exposure results in a threefold increase in LHRH-positive cells in the diencephalon, and female rats exposed to the gas also do not exhibit normal estrous cycles. The significant increases in the LHRH concentration in the POA of N_2O-exposed animals observed in the present study is in general agreement with our previous immunocytochemical study.[9] Taken together, these results indicate that the antigonadotropic effects of N_2O may be centrally mediated. We did not observe any significant changes in LHRH in the MBH. If LHRH release was inhibited by N_2O, an increase in LHRH at the site of its release, which is the MBH, would be expected. The reason for the selective increase in LHRH only in the POA of N_2O-exposed animals is not known. It is possible that N_2O may inhibit posttranslational processing of the precursor form of LHRH, resulting in elevations of various precursor fragments of LHRH within the cell bodies, mainly located in the POA. As our LHRH antiserum was directed toward the internal sequences of LHRH, the possibility that precursor forms of LHRH, in addition to the mature form, were recognized in our radioimmunoassay cannot be ruled out. Future studies in our laboratories will focus on the effects of N_2O on the posttranslational processing events associated with LHRH. It also is not clear whether N_2O effects directly the LHRH cells or whether its effects are mediated via other neural networks that interact with the LHRH system (for example, the endogenous opioid neurons).

N_2O has specific effects on the endogenous opioids met-enkephalin and β-endorphin. We found that 30% N_2O resulted in significant elevations of met-enkephalin in the brainstem and β-endorphin in the pituitary (intermediate–anterior lobes combined). Exposure to 75% N_2O for 1 hr has been shown to elevate met-enkephalin in cerebrospinal fluid (CSF) collected from the cerebral ventricle.[25] Morris and Livingston[26] did not find any difference in the concentrations of met-enkephalin in the following areas in rats exposed to either 1 or 18 hr of 80% N_2O: medial thalamus, dorsal raphe, peri-aqueductal grey, raphe magnus, and locus coeruleus. These two studies are not necessarily in conflict. An increase in the CSF titer of an opioid peptide does not necessarily result from an overall increase in the entire brain content of the peptide. Any increases in a peptide in discrete areas of the brain also may not parallel changes in the CSF concentration of the same peptide, as most peptide transmitters are inactivated quickly and at the site of secretion.

It is interesting to note that 30% N_2O increased β-endorphin significantly in the intermediate–anterior lobes of the pituitary but did not alter its concentration in the diencephalon. Previous studies in other laboratories have shown that 30% N_2O did not alter plasma β-endor-

Figure 4. Endorphin content in the intermediate–anterior lobes of the pituitary of rats exposed to either 30% N_2O or air. Open star indicates statistical significance at $P < 0.05$.



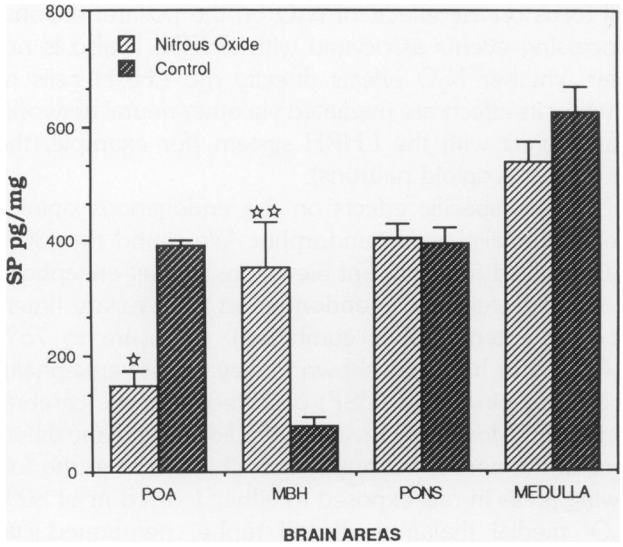


Figure 5. Effect of exposure to either 30% N_2O or air on substance P (SP) in the POA, MBH, and brainstem areas. Concentrations were significantly lower in the POA ($P < 0.05$) and higher in the MBH ($P < 0.01$) of N_2O-exposed animals.

phin in humans.[27] However, at higher concentrations (60% and 80%), N_2O was effective in significantly elevating β-endorphin in the MBH and peri-aqueductal grey of rats.[28] These higher concentrations of N_2O also were effective in increasing the release of β-endorphin from cultured hypothalamic neurons.[29] In agreement with our results, 30% N_2O had no effect on β-endorphin in the MBH.[28,29] There are no previous reports on the effect of N_2O on β-endorphin in the pituitary. The significant increases in pituitary β-endorphin observed in our study may be due to increased synthesis or decreased release of β-endorphin. The altered β-endorphin activity in the pituitary gland by N_2O is probably related to the abnormal estrous cycles exhibited by the animals. A direct opioid regulation of the pituitary gonadotropin has previously been shown.[30]

The effects of N_2O on brain SP concentrations are reported for the first time in this study. Substance P has been implicated in the regulation of gonadotropins.[17,31–33] That SP neurons may have a direct effect on hypothalamic neurons by synaptic input has been demonstrated previously.[34] Gonadal steroids have been shown involved in the regulation of hypothalamic SP, implicating SP in the regulation of gonads.[24,35] The concentration of SP also has been shown significantly decreased in the POA during proestrous and elevated during diestrous, indicating a role for this neuropeptide in estrous cycle events.[36] We have consistently observed an inverse relationship between LHRH and SP concentrations in brain under varying steroidal conditions.[24] It is interesting to note that the results of this study also indicate elevated LHRH but decreased SP in the POA of N_2O-exposed rats. Although the exact nature of the interaction of SP and LHRH remains to be elucidated, it is tempting to speculate that the N_2O-induced changes in SP and LHRH are partly responsible for the altered reproductive status induced by the gas.

The significance of increased met-enkephalin in the brainstem area of N_2O-exposed animals is not known but may be related to the anesthetic and analgesic effects induced by N_2O. Preliminary studies in our laboratory indicate that exposure to 30% N_2O results in elevations of pre-proenkephalin mRNA levels in the cortex and thalamic areas of the rat but not in the brainstem. These results indicate the need to focus future investigations on the effects of N_2O on, not only the transcriptional control of enkephalin synthesis, but also the posttranslational processing and release mechanisms of the peptide.

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