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Tolerance to Nitrous Oxide Analgesia in Rats and Mice

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The purpose of these experiments was to characterize the nature of tolerance to the analgesic action of nitrous oxide. Analgesia was assessed in rats using a tail-flick latency test and in mice using an abdominal constriction test. Rats and mice were exposed to nitrous oxide, 75 per cent, the balance oxygen, continuously for 16–18 hours. On re-exposure to nitrous oxide 30 min later, these animals were found tolerant to nitrous oxide in that the analgesic response was decreased by at least 50 per cent. Animals tolerant to nitrous oxide were not tolerant to morphine. Morphine (0.25–1.5 mg/kg) produced equal degrees of analgesia in control and nitrous oxide-tolerant mice and rats. In contrast, rats made tolerant to morphine by repeated daily injections of as much as 400 mg/kg subcutaneously or by subcutaneous implantation of morphine pellets (75 mg, twice) showed a decreased analgesic response to nitrous oxide. Thus the cross-tolerance between nitrous oxide and morphine appears unique in that it is unidirectional. (Key words: Analgesics, narcotic: morphine, tolerance. Anesthetics, gases, nitrous oxide.)

RECENTLY, we presented evidence for an analgesic action of nitrous oxide in mice and rats.[1,2] Methods for the exposure of rodents to nitrous oxide were described, along with the concurrent estimation of analgesia. In characterizing the nature of the analgesic action of nitrous oxide there are several lines of evidence suggesting the participation of an opiate-like action. First, nitrous oxide, like opiates, is an analgesic and euphoriant substance. Second, in animal studies naloxone partially decreases the analgesia produced by nitrous oxide. Third, mice tolerant to morphine are also cross-tolerant to nitrous oxide-induced analgesia.

Tolerance occurs to many of the effects of narcotic analgesics and depressants. Tolerance to nitrous oxide analgesia following prolonged exposure to the gas has been found in animals[2] and man.[3] It has not been determined whether tolerance to nitrous oxide conveys tolerance to opiates.

Method

Analgesia was assessed in rats using the tail-flick latency test.[2] Male rats, weighing 200–300 g, were placed in individual metal chambers each having a volume of 400 ml and a close-fitting plastic cover. The tails of the rats protruded through a rubber-encased hole at one end of the chamber. Air or a nitrous oxide–oxygen mixture was delivered to the chambers through a manifold at a total flow of 10 l/min. After the rat had breathed air for 5–15 min, control tail-flick latency was measured as the time in seconds for the rat to remove its tail from a thermal stimulus. The thermal stimulus intensity was adjusted so that untreated rats had a latency of 3–5 sec. This stimulus intensity was left constant for all experiments.

The rats were then exposed to the nitrous oxide–oxygen mixture for 15 min and tail-flick latency redetermined. The duration of thermal stimulus was limited to 10 sec. Calculation for per cent analgesia was

$$\frac{\text{Experimental tail-flick time} - \text{control tail-flick time}}{10 - \text{control tail-flick time}} \times 100$$

An additional measure of analgesia is referred to as "seconds of analgesia": the experimental tail-flick time minus the control tail-flick time.

Analgesia was assessed in mice by use of the abdominal constriction test.[2] Mail Swiss-Webster mice, weighing 20–25 g, received intraperitoneal injections of phenyl-p-benzoquinone,¶ 0.01 ml/g of a 0.025 per cent solution in ethanol, 5 per cent and were placed individually in small wire cages in a clear plastic box (14-l volume) with a cover and gas inlet and outlet ports at either end. Air or the nitrous oxide–oxygen mixture was delivered to the box through calibrated flowmeters at a total flow of 10 l/min. Results are based on the number of abdominal constrictions per group of five mice observed during the five-minute interval from 10 to 15 min after the injection. Each group of animals was used only once, breathing air or the nitrous oxide–oxygen mixture. Analgesia in per

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¶ Eastman Kodak Company.

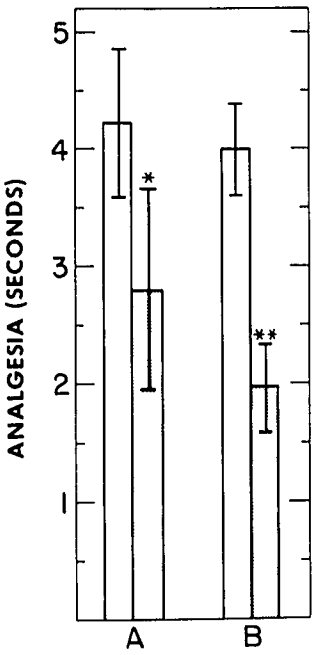

FIG. 1. Rats tolerant to morphine are cross-tolerant to nitrous oxide. Long-Evans rats (*A*, n = 10 per group) or Sprague-Dawley rats (*B*, n = 35 per group) were made tolerant to morphine as described in Methods. Open bars represent controls, darkened bars, morphine-treated rats. Nitrous oxide (80 per cent) was administered to all animals and analgesia measured by tail-flick latency. Results are means ± SE. *$P < .05$ compared with nontolerant rats. **$P < .005$ compared with nontolerant rats.

cent was calculated as

$$\frac{\text{Control constrictions} - \text{treatment constrictions}}{\text{Control constrictions}} \times 100$$

To study the development of tolerance to nitrous oxide, rats were placed in rectangular Plexiglas® cages (45 × 20 × 19 cm) each having a cover and gas inlet and outlet ports. Soda lime was layered on the bottom of the chamber to absorb CO_2 and covered with cotton gauze. The grid floor of the cage was suspended above the soda lime. Water was supplied through an inlet port and food was placed on the floor of the cage. The nitrous oxide–oxygen mixture was humidified by bubbling through water and delivered at a rate of 10 l/min. As controls, groups of rats were placed in identical cages, but humidified compressed air was delivered instead of the nitrous oxide–oxygen mixture. Exposures of 16–18 hours were followed by 30 min exposure to room air prior to testing for analgesia. This was done to allow time for the elimination of nitrous oxide. Similar conditions were used for the chronic exposure of mice.

Rats were chronically treated with morphine to produce tolerance. Male Long-Evans rats received injections of morphine sulfate** twice a day for 14 days in increasing doses until a total daily dose of 400 mg/kg was achieved. Control rats received injections of saline solution. Rats received their final doses about 14 hours before testing for analgesia. A second method for making rats tolerant to morphine was by pellet implantation. Male Sprague-Dawley rats each received two 5-mg/kg injections of morphine subcutaneously as a priming dose on day one. A pellet containing 75 mg of morphine base prepared as previously described[4] was implanted subcutaneously under light ether anesthesia on day 2 and another on day 3. Control rats received placebo pellets. Studies for analgesia and tolerance were conducted on day 5 following removal of pellets. Tolerance to morphine was confirmed by demonstrating the lack of analgesic action of morphine. Testing for analgesia induced by morphine was done 30 min following subcutaneous injections. All tests for analgesia were performed between 9:00 A.M. and 2:00 P.M. Doses of morphine are expressed as the salt.

Because a 10-sec cutoff is used in the analgesic tail-flick tests, data were analyzed by nonparametric tests: the Mann-Whitney U-test and median tests.[5] $P < .05$ was accepted as significant. Results are expressed as mean ± standard error of the mean.

Results

In the Long-Evans rats receiving daily injections of morphine in increasing doses for 14 days, tolerance to morphine was shown by testing for morphine-induced analgesia. In control rats, morphine 1.5 mg/kg, subcutaneously, produced 100 per cent analgesia in 30 min, as evidenced by results of the tail-flick test. Rats chronically treated with morphine showed only 32 per cent analgesia (n = 5 for each group, $P < .005$). In additional groups of rats (n = 10 for each group), 80 per cent nitrous oxide produced 74 per cent analgesia (or 4.2 sec of analgesia) in saline-treated control rats, whereas it produced only 55 per cent analgesia (2.8 sec of analgesia) in morphine-tolerant rats (fig. 1*A*).

These results were confirmed using larger groups of Sprague-Dawley rats made tolerant to morphine by implantation of morphine pellets. Morphine, 1.5 mg/kg, subcutaneously, produced 70 per cent analgesia in 30 min in sham-operated control rats. The same dose of morphine injected into morphine pellet-implanted rats gave only 13 per cent analgesia (n = 5 for each group, $P < .01$). The analgesic action of nitrous oxide was again decreased in morphine pellet-implanted rats compared with controls (n = 35 for each group, $P < .005$) (fig. 1*B*).

Rats exposed to nitrous oxide (75 per cent) or air for 16 hours showed similar tail-flick latencies to the thermal stimulus when tested 30 min after removal from their exposure chambers. The five rats exposed to nitrous oxide had an average tail-flick latency of 4.2 ± 0.3 sec. Control rats breathing room air over-

** Merck and Company, Inc.

night had a tail-flick latency of 4.2 ± 0.2 sec. Thus, there was no residual analgesic effect of nitrous oxide in rats. However, tolerance to nitrous oxide occurred following overnight exposure (fig. 2, *left panel*). Nitrous oxide, which produced 63 per cent analgesia (3.9 sec of analgesia) in control rats, produced only 23 per cent analgesia (1.7 sec of analgesia) in rats previously exposed to nitrous oxide.

Morphine (1.5 mg/kg) was tested in another set of the air-exposed and nitrous oxide-exposed rats. Morphine was equianalgesic in nitrous oxide-exposed and air-exposed rats (fig. 2, *right panel*). Thus, rats tolerant to nitrous oxide are not cross-tolerant to morphine.

In mice exposed to 75 per cent nitrous oxide for 14–16 hours, the analgesic action of nitrous oxide was also decreased, whereas the analgesic action of morphine was not (table 1). In nitrous oxide-exposed mice, treatment with naloxone (10 mg/kg) did not produce any sign of opiate-like withdrawal such as jumping, escape behavior, or acute weight loss from urination and defecation.

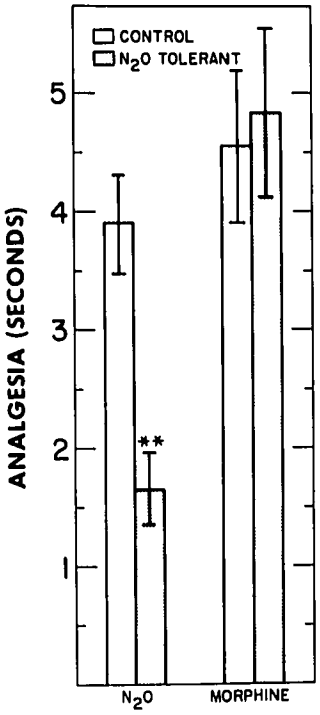

FIG. 2. Rats tolerant to nitrous oxide are not tolerant to morphine. Rats were exposed for 16 hours to room air (open bars) or 75 per cent nitrous oxide (darkened bars). After breathing room air for 30 min, rats were tested for their analgesic responses to nitrous oxide (80 per cent) or morphine, 1.5 mg/kg, sc, administered 30 min prior to testing. Analgesia was measured by tail-flick latency. Results are means ± SE obtained from at least five animals for each treatment. $**P < .001$ compared with control rats.

Discussion

Analgesic studies with nitrous oxide in experimental animals are a recent development.[1,2] We elected to study nitrous oxide at normal atmospheric pressure since it is under this condition that it is usually used. Rats made tolerant to morphine by implantation of morphine pellets or repeated injections of morphine show also a decreased analgesic response to nitrous oxide. This decreased response to nitrous oxide in morphine-tolerant mice is only partial. We used two strains of rats and observed decreased analgesic responses in both. These results extend our original observation that mice chronically treated with morphine are partially resistant to the analgesic action of nitrous oxide.[1]

Tolerance to an opiate conveys cross-tolerance to other opiates. Likewise, tolerance to depressants conveys cross-tolerance to other depressants. The cross-tolerance described in this study is unique in that tolerance to an opiate (morphine) was associated with a cross-tolerance to nitrous oxide. But neither rats nor mice tolerant to nitrous oxide were tolerant to morphine. Thus, the cross-tolerance that does occur in morphine-tolerant animals is only unidirectional.

One must be cautious in the interpretation of these findings. One factor to consider in the development of nitrous oxide tolerance is that mice and rats were exposed to the gas for only about 16–18 hours. It is possible that this is not long enough for detectable cross-tolerance to narcotics to develop. Arguing against this explanation, however, are data showing that the adaptive mechanisms mediating tolerance to and dependence on morphine can develop very rapidly.[6] Thus, the tolerance to nitrous oxide but lack of cross-tolerance to morphine in animals exposed to nitrous oxide for 16 hours is not readily explained by the relatively short exposure to nitrous oxide.

One hypothesis that could explain the analgesic action of nitrous oxide and tolerance involves the en-

TABLE 1. Effect of Morphine or Nitrous Oxide in Mice Chronically Exposed to Nitrous Oxide

Drug, mg/kg (n)	Chronic N_2O Exposure (16 Hours)	Analgesia (Per Cent)
Nitrous oxide (15)	—	53 ± 10
Nitrous oxide (15)	+	6 ± 6*
Morphine, 0.25 (10)	—	19–25 (range)
Morphine, 0.25 (10)	+	14–17 (range)
Morphine, 0.75 (20)	—	58 ± 9
Morphine, 0.75 (20)	+	65 ± 14

Mice were exposed to air or nitrous oxide (75 per cent) for 16 hours and then allowed to remain in room air for 30 min before testing with nitrous oxide (80 per cent) or morphine. (n) is the number of mice pooled into groups of five for the analgesic test, using the abdominal constriction test. The observer was unaware of the drug treatments, and all mice were fasted during the experiment. Values are means ± SE except where noted.

* Differs significantly from value for mice acutely exposed to nitrous oxide, $P < 0.05$.

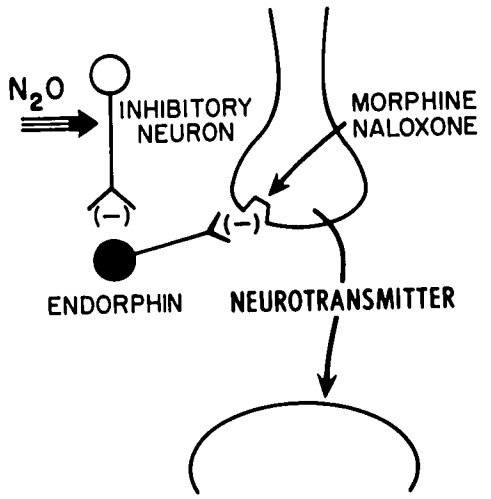

FIG. 3. Proposed mechanism of analgesic action of nitrous oxide. Nitrous oxide administration results in release of endorphin, which acts at opiate receptors in the central nervous system.

dogenous opiate system (fig. 3). Nitrous oxide may release one of these substances (endorphins), which act on opiate receptors in the central nervous system, inhibiting neurotransmission in neural pathways associated with pain. Naloxone blocks the action of nitrous oxide in this model by antagonizing the action of endogenous opiates.[2] The relatively low ability of naloxone to antagonize nitrous oxide compared with its ability to antagonize narcotics is remarkably similar to the lower potency of naloxone in antagonizing endogenous opiates compared with narcotics.[7] Since naloxone alone usually has no hyperalgesic effect, the endogenous opiate system may normally not be active or be under tonic inhibition. One possibility is that nitrous oxide may decrease this inhibition, resulting in endorphin release and analgesia. Other central nervous system depressants may produce similar effects, but since they are more potent than nitrous oxide, many other neuronal systems are also depressed and anesthesia is the result. Tolerance to nitrous oxide may develop not at opiate receptors but as endorphin stores are exhausted or as the ability of nitrous oxide to depress inhibiting neurons and allow the release of endorphins wanes. Thus, with nitrous oxide tolerance, exogenous opiates would act directly on opiate receptors and produce analgesia. We are currently examining the effect of nitrous oxide on endorphin disposition in the brain.

There is no question that nitrous oxide is an abusable substance. Self-administration of nitrous oxide has recently been demonstrated in animals.[8] In man there is clear evidence that tolerance can develop to nitrous oxide analgesia,[3] and it has been suggested that nitrous oxide might prevent narcotic withdrawal.[9] Recently, Clark and Yang reported that naloxone partially decreased the analgesic action of nitrous oxide in man.[10]

It should be emphasized that we are not suggesting that the pharmacologic effects of nitrous oxide are explained entirely by an action involving opiate-like mechanisms or endorphins. Indeed, even if analgesia induced by nitrous oxide involves endorphins, our data suggest only a partial involvement, since pretreatment with naloxone and morphine tolerance only partially decrease the effects of nitrous oxide.[1,2,11] Recently, Smith *et al.*[12] reported that in mice, naloxone, 2 and 16 mg/kg, intraperitoneally, did not significantly change the ED_{50} values of nitrous oxide, tested by righting reflex, suggesting that mechanisms of nitrous oxide-induced analgesia and loss of righting reflex (under hyperbaric conditions) may not be the same.

References

1. Berkowitz BA, Ngai SH, Finck AD: Nitrous oxide analgesia: Resemblance to opiate action. Science 194:967–968, 1976
2. Berkowitz BA, Finck AD, Ngai SH: Nitrous oxide analgesia: Reversal by naloxone and development of tolerance. J Pharmacol Exp Ther 203:539–547, 1977
3. Whitman JG, Morgan M, Hall GM, et al: Pain during continuous nitrous oxide administration. Br J Anaesth 48: 425–428, 1976
4. Berkowitz BA, Cerreta KV, Spector S: The influence of physiologic and pharmacologic factors on the disposition of morphine as determined by radioimmunoassay. J Pharmacol Exp Ther 191:527–534, 1974
5. Freund JE: Modern Elementary Statistics. Second edition. Englewood Cliffs, N. J., Prentice Hall, 1961
6. Way EL, Loh HH, Shen FH: Simultaneous quantitative assessment of morphine tolerance and physical dependence. J Pharmacol Exp Ther 167:1–8, 1964
7. Lord JA, Waterfield A, Hughes J, et al: Endogenous opioid peptides: Multiple agonists and receptor. Nature 267: 495–499, 1977
8. Wood RW, Grubman J, Weiss B: Nitrous oxide self-administration by the squirrel monkey. J Pharmacol Exp Ther 202: 491–499, 1977
9. Kripke BJ, Hechtman HB: Nitrous oxide for pentazocine addiction and for intractable pain: Report of a case. Anesth Analg (Cleve) 51:520–526, 1972
10. Clark WC, Yang JC: Naloxone reversal of nitrous oxide hypalgesia in man. Pain Abstr 2nd World Congress on Pain 1:14, 1978
11. Hynes MD, Berkowitz BA: Nitrous oxide stimulation of locomotor activity: Evidence for an opiate-like behavioral effect. J Pharmacol Exp Ther 209:304–308, 1979
12. Smith RA, Wilson M, Miller KW: Naloxone has no effect on nitrous oxide anesthesia. ANESTHESIOLOGY 49:6–8, 1978