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Nitrous Oxide Produces Antinociceptive Response via α_{2B} and/or α_{2C} Adrenoceptor Subtypes in Mice

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Background: **Opiate receptors in the periaqueductal gray region and α_2 adrenoceptors in the spinal cord of the rat mediate the antinociceptive properties of nitrous oxide (N_2O). The availability of genetically altered mice facilitates the detection of the precise protein species involved in the transduction pathway. In this study, the authors establish the similarity between rats and mice in the antinociceptive action of N_2O and investigate which α_2 adrenoceptor subtypes mediate this response.**

Methods: **After obtaining institutional approval, antinociceptive dose–response and time–course to N_2O was measured in wild-type and transgenic mice (D79N), with a nonfunctional α_{2A} adrenoceptor using tail-flick latency. The antinociceptive effect of N_2O was tested after pretreatment systemically with yohimbine (nonselective α_2 antagonist), naloxone (opiate antagonist), L659,066 (peripheral α_2-antagonist) and prazosin (α_{2B}- and α_{2C}-selective antagonist). The tail-flick latency to dexmedetomidine (D-med), a nonselective α_2 agonist, was tested in wild-type and transgenic mice.**

Results: **N_2O produced antinociception in both D79N transgenic and wild-type litter mates, although the response was less pronounced in the transgenic mice. Antinociception from N_2O decreased over time with continuing exposure, and the decrement was more pronounced in the transgenic mice. The antinociceptive response could be dose dependently antagonized by opiate receptor and selective α_{2B}-/α_{2C}-receptor antagonists but not by a central nervous system-impermeant α_2 antagonist (L659,066). Whereas dexmedetomidine exhibited no antinociceptive response in the D79N mice, the robust antinociceptive response in the wild-type litter mates could not be blocked by a selective α_{2B}-/α_{2C}-receptor antagonist.**

Conclusion: **These data confirm that the antinociceptive response to an exogenous α_2-agonist is mediated by an α_{2A} adrenoceptor and that there appears to be a role for the α_{2B}- or α_{2C}-adrenoceptor subtypes, or both, in the analgesic response to N_2O. (Key words: Analgesia; opiate receptors; tail flick.)**

ALMOST 200 years ago, Sir Humphrey Davy[1] first demonstrated the analgesic properties of nitrous oxide (N_2O); however, the mechanism for this action still has not been defined. Inhalation of 20–25% N_2O in oxygen can produce an analgesic effect equivalent to that produced by 15 mg morphine sulfate in humans[2] and similar to morphine, N_2O analgesia is in part reversed by opiate-receptor antagonists in humans and animals.[3–7] Furthermore, there is unilateral cross-tolerance between morphine and N_2O.[4,8]

Based on these observations, a mechanism of action for N_2O has been proposed in which opiate receptors are activated through the release of endogenous ligands. Neurochemical studies have supported such a mechanism.[9–12] The periaqueductal gray has long been known to be an important site for the analgesic action of opiates (Cf)[13]; therefore, we[14] and others[15] demonstrated that the analgesic properties of N_2O could be blocked by the discrete introduction of opiate antagonists directly into the periaqueductal gray. Furthermore, because Camarata *et al.*[16] demonstrated that opiate-induced analgesia at the periaqueductal gray could be blocked by intrathecal administration of α_2 antagonists; we followed this up by demonstrating that the antinociceptive effects of N_2O too could be prevented by blockade of α_2 adrenoceptors in the spinal cord.

Molecular genetic cloning studies in humans, rats, and mice have shown that, in each of these species three genes on separate chromosomes encode distinct α_2-adrenergic receptor subtypes. Pharmacologic studies have defined four subtypes named α_{2A}, α_{2B}, α_{2C}, and α_{2D},

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with the α_{2D} representing a rodent species homologue of human α_{2A} (for review see reference [17]). Radiolabeled ligand and *in situ* hybridization studies with mRNA probes reveal that the receptor subtypes are nonhomogeneously distributed in the central nervous system.[18–26] In the central nervous system, the α_{2A} subtype is the most prevalent and ubiquitous of the three, whereas α_{2B} is only present in a few discrete sites, principally the thalamic nuclei, and then in very small amounts. Because of the absence of selective pharmacologic probes, we[27] and others[28,29] resorted to genetically altered animals to ascribe the various properties of α_2 agonists to the different receptor subtypes. Using transgenic mice (D79N; provided by Dr. Lee Limbird) in which a single amino acid residue at position 79 has been mutated from aspartic acid (D) to asparagine (N) to render the α_{2A}-receptor subtype dysfunctional,[30] we reported that the antinociceptive properties of a nonselective α_2 agonist were mediated by the α_{2A}-adrenoceptor subtype[27]. We now investigate whether the α_{2A}-adrenoceptor subtype is exclusively responsible for the antinociceptive properties of N_2O.

Methods

The experimental protocol was approved by the Animal Care and Use Committee at the Veterans Administration Palo Alto Health Care System. Male 129/svj wild-type mice (Jackson Labs, Bar Harbor, ME) and D79N transgenic mice (bred at Vanderbilt University, Nashville, TN) weighing 20–30 g (8–10 weeks old) at the time of testing were used. A total of 152 mice were used. Each mouse may have been tested on more than one occasion with a period of 1 week allowed to elapse between successive testings. (In preliminary studies we established that responsiveness does not differ week to week.)

Antinociceptive Testing. The antinociceptive response was assessed using an analgesiometer for measurement of the tail-flick latency (TFL) response. A high-intensity light was focused on the middle third of the mouse's tail, and the time for the mouse to move its tail out of the light beam was automatically recorded (Tail-Flick Apparatus; Columbus Instruments, Columbus, OH) and referred to as TFL. A different patch of the tail was exposed to the light beam on each trial to minimize the risk of tissue damage. The animals were placed on the heating blanket to maintain body and tail temperature during the experiment. A cut-off time of 10 s was predetermined, at which time the trial was discontinued if no response occurred. Each TFL data point consisted of an average of two trials on an individual animal. From the TFL the percent maximal possible antinociceptive effect (%MPE) was calculated as follows:

$$- \text{(postdrug latency)} - \text{(basal latency)} \,/\, \text{(cut-off latency)} - \text{(basal latency)} \times 100\%.$$

Our decision to use this "spinal reflex" to investigate the antinociceptive response to N_2O is predicated by its widespread application and validation to define the pharmacology of spinally mediated nociception. Alterations in these reflexes may also reflect changes in the activity of supraspinally located neurons that regulate the afferent transmission of nociceptive information by means of direct or indirect projections to the dorsal horn of the spinal cord (for review see reference [31]).

Gas Exposures. All gas exposures were performed in an acrylic chamber (81 × 43 × 34 cm) with a sliding door on one side (for insertion of the mice). This airtight chamber was large enough to contain the analgesiometer device. Fresh test gases (10 l/min) were introduced into the chamber *via* an inflow port, circulated throughout the chamber by a small fan, and purged by vacuum set to aspirate at the same rate as the fresh gas inflow. Oxygen concentration in the chamber was maintained between 22 and 45%, whereas N_2O concentration was varied between 0, 25, 50, and 75% by adjusting the flow rates of N_2O, air, and nitrogen (AirLiquide, Houston, TX). Gas concentrations were measured continuously and flow rates were adjusted appropriately to maintain the desired concentrations.

Experimental Schedule

To determine the dependence of N_2O-induced antinociception on α_2 adrenoceptors, cohorts of mice (n = 8) were pretreated with yohimbine (Sigma Chemical, St Louis, MO), 1 and 2 mg/kg intraperitoneally 30 min before 70% N_2O exposure, or cohorts of mice (n = 6) were treated with atipamezole (Orion-Farmos, Turku Finland), 1 mg/kg 15 min before dexmedetomidine (Orion-Farmos) 100 μg/kg intraperitoneally.

To determine the dependence on peripheral α_2 adrenoceptors, cohorts of mice (n = 8) were pretreated with L659,066 (Merck, Sharp and Dhome Laboratories, Rahway, NJ), 1 and 10 mg/kg intraperitoneally 30 min before N_2O exposure, or cohorts of mice (n = 6) were treated with intraperitoneal L659,066, 10 mg/kg 15 min before intraperitoneal dexmedetomidine 100 μg/kg.

To determine the dependence of α_{2A}- or α_{2B}-adreno-

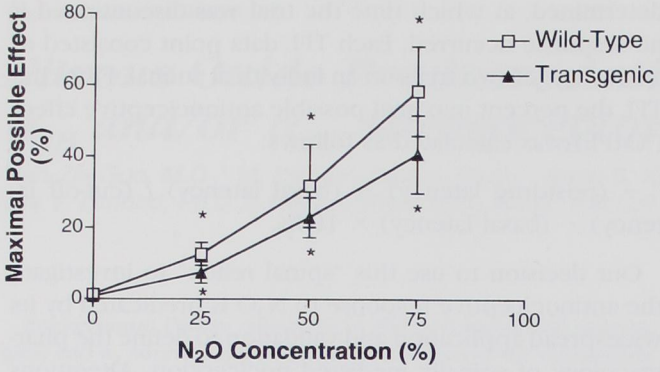

Fig 1. Dose–response relationship of N_2O analgesic effect in wild-type and α_{2A} transgenic mice. Different groups of mice (n = 8 per group) were exposed for 30 min to N_2O (25%, 50%, and 75%) or to air in an enclosed chamber. Tail-flick latency was then measured. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. When the two groups of mice are analyzed by two-way analysis of variance, the antinociceptive response curves are statistically significantly different. $P < 0.0001$.

ceptor subtypes, or both, cohorts of mice (n = 8) were pretreated with prazosin (Sigma Chemical), 1, 5, and 10 mg/kg intraperitoneally 30 min before 70% N_2O exposure, or cohorts of mice (n = 6) were treated with prazosin, 10 mg/kg intraperitoneally 15 min before intraperitoneal dexmedetomidine, 100 μg/kg.

At the doses used, the antagonists had no independent effect on TFL.

Statistical Analysis. In most cases, data were analyzed using a single-factor analysis of variance and expressed as a mean ± standard error of the mean. To assess whether the response curves for the effect of concentration and time of exposure were different, two-way analysis of variance was performed.

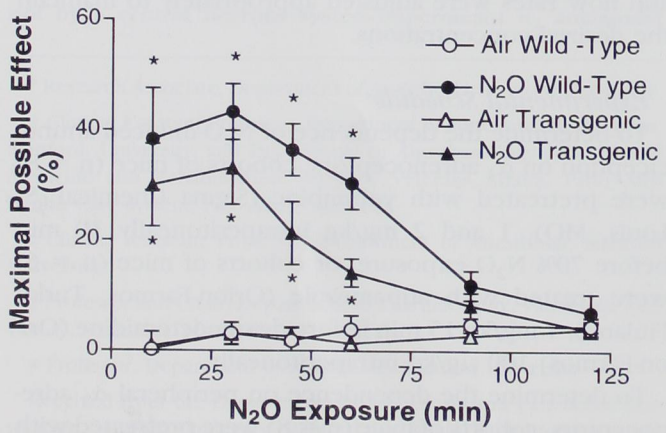

Fig 2. Time course of N_2O analgesic effect in wild-type and α_{2A} transgenic mice. Mice (n = 8 per group) were exposed to 70% N_2O or to air in an enclosed chamber. Tail-flick latency was measured at a scheduled time. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. When the two groups of mice are analyzed by two-way analysis of variance, the antinociceptive time–course curves are statistically significantly different. $P < 0.01$.

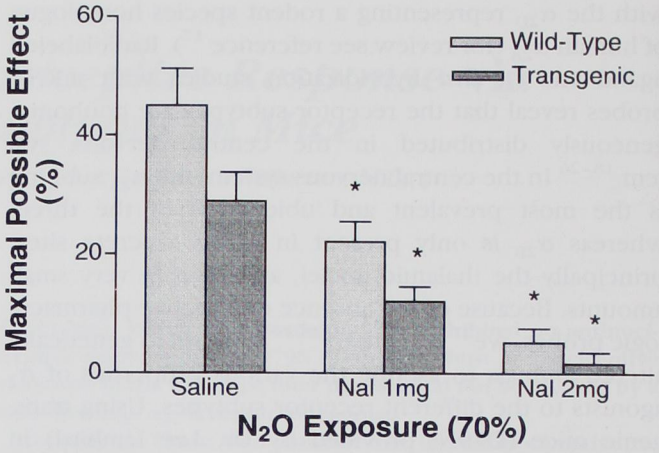

Fig 3. Effect of naloxone on analgesic response to N_2O. Three groups of wild-type and α_{2A} transgenic mice (n = 8 per group) were administered saline and naloxone, 1.0 and 2 mg/kg intraperitoneally, then immediately exposed to 70% N_2O. Tail-flick latency was measured before and 30 min after injection. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. Naloxone effect did not differ between wild-type and transgenic mice.

Results

Exposure to N_2O produced a dose-dependent antinociceptive response in both the wild-type and the transgenic mice (fig. 1). The time-course of antinociception during continuous exposure to 70% N_2O (fig. 2) reveals a maximum response at 30 min, which gradually returned to baseline over the next 60 min. When individual concentrations and time points were compared, no significant difference was noted between transgenic and wild-type mice by single-factor analysis of variance. However when data from all concentrations were simultaneously analyzed by two-factor analysis of variance, the response of the transgenic animals was found to be less than observed in the wild type. In both types of mice, the antinociceptive response was blocked dose dependently by the opiate antagonist naloxone (fig. 3) and the nonselective α_2 antagonist yohimbine (fig. 4). However, the peripherally active α_2 antagonist L659,066 (fig. 5) was without effect in either the transgenic or the wild-type mice. Prazosin, the selective α_{2B} and α_{2C} antagonist, dose dependently blocked the antinociceptive effect in both the wild-type and the transgenic mice (fig. 6).

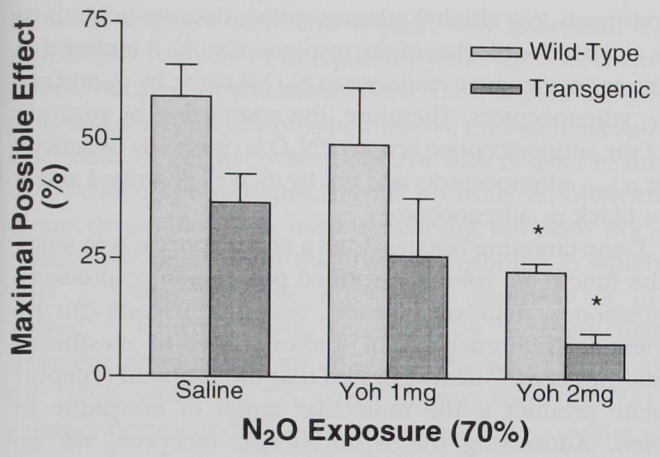

Fig 4. Effect of yohimbine on analgesic response to N_2O. Three groups of wild-type and α_{2A} transgenic mice (n = 8 per group) were administered intraperitoneally by saline and yohimbine, 1.0 and 2 mg/kg, then immediately exposed to 70% N_2O. Tail-flick latency was measured before and 30 min after injection. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. Yohimbine effect did not differ between wild-type and transgenic mice.

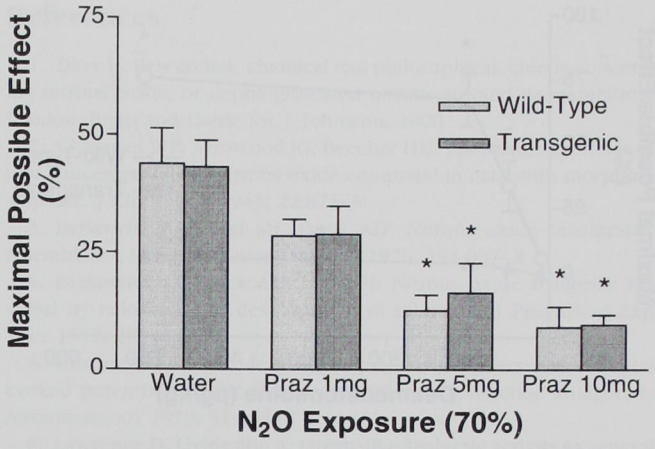

Fig 6. Effect of prazosin on analgesic response to N_2O. Four groups of wild-type and α_{2A} transgenic mice (n = 8 per group) were administered distilled water and prazosin 1.0, 5.0, and 10 mg/kg intraperitoneally, then immediately exposed to 70% N_2O. Tail-flick latency was measured before and 30 min after injection. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. Prazosin effect did not differ between wild-type and transgenic mice.

Dexmedetomidine, the potent nonselective α_2 agonist, exhibits a dose-dependent antinociceptive effect in wild-type animals (fig. 7A); unlike N_2O (fig. 1), dexmedetomidine does not produce antinociception in transgenic animals (fig. 7A). The antinociceptive effect of dexmedetomidine in wild-type mice was not blocked by doses of prazosin (fig. 7B), which significantly attenuated the antinociceptive response to N_2O (fig. 6). Similarly, the antinociceptive effect of dexmedetomidine in wild-type mice was not blocked by L659,066, the peripheral α_2 antagonist.

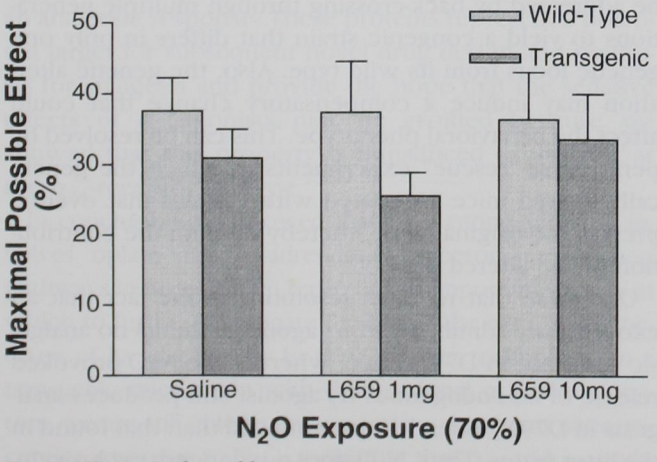

Fig 5. Effect of L659,066 on analgesic response to N_2O. Three groups of wild-type and α_{2A} transgenic mice (n = 8 per group) were administered saline and L659,066, 1.0 and 10 mg/kg intraperitoneally, then immediately exposed to 70% N_2O. Tail-flick latency was measured before and 30 min after injection. Data are expressed as mean ± SEM.

Discussion

From these data we may deduce that, similar to the rat,[32] the antinociceptive response to N_2O in mice is dependent on opiate and α_2-adrenergic receptor pathways. In D79N mice, a genetically altered strain with dysfunctional α_{2A} adrenoceptors, N_2O can still exert a significant antinociceptive response. Because this antinociceptive response can be blocked by a nonselective α_2 antagonist, these data indicate that receptor subtypes other than α_{2A} transduce the N_2O effect. This is supported by the experiment in which prazosin (α_{2B}- and α_{2C}-subtype antagonist with extremely low affinity for the α_{2A} subtype)[33,34] blocked the antinociceptive effect of N_2O. Although dexmedetomidine and N_2O both produce their antinociceptive responses *via* α_2 adrenoceptors (blocked by either yohimbine [fig. 4] or atipamezole [fig. 7B]), there are two major differences. Prazosin does not attenuate the antinociceptive response to dexmedetomidine (fig. 7B), whereas it dose dependently blocks the antinociceptive response to N_2O (fig. 6). Secondly, dexmedetomidine is ineffective in D79N transgenic mice, whereas our current report shows that the response to N_2O is still present in these mice.

Definition of the molecular components involved in a

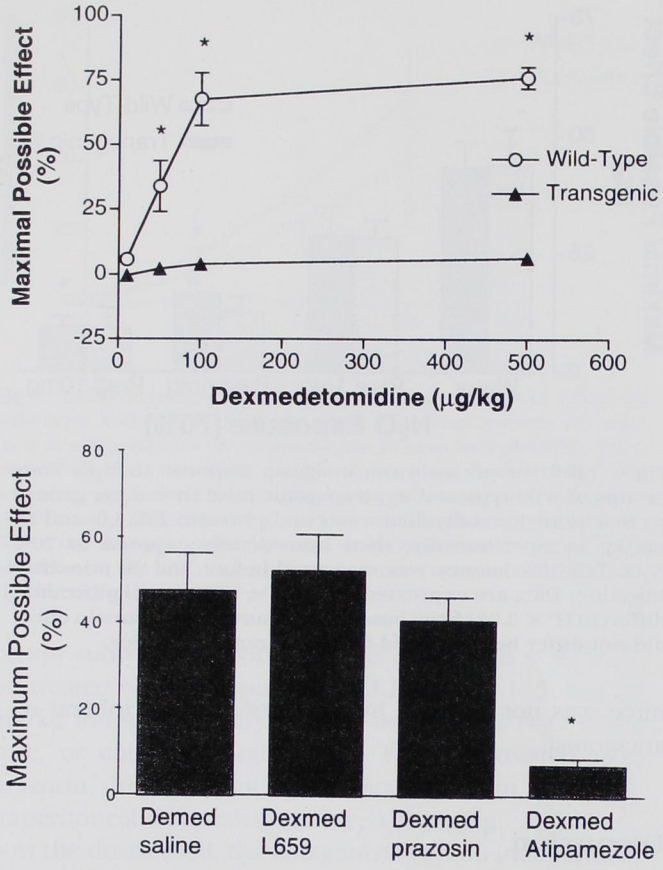

Fig 7. (*A*) The analgesic effect of dexmedetomidine in wild-type and α_{2A} transgenic mice. A cumulative (intraperitoneal) dexmedetomodine dose–antinociceptive response was performed in the two species of mice (n = 9 per group) using tail-flick latency response 30 min after dexmedetomidine injection. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from baseline measurement. (*B*) The ability of various antagonists to block the analgesic effect of dexmedetomidine was tested in four cohorts of wild-type mice (n = 6). Cohorts were pretreated 15 min before intraperitoneal dexmedetomidine (100 μg/kg) with either intraperitoneal saline, (L659,066 10 mg/kg), intraperitoneal prazosin, (10 mg/kg), or intraperitoneal atipamezole (1 mg/kg). Tail flick latency was measured before and 30 min after dexmedetomidine injection. Data are expressed as mean ± SEM. * = significantly different ($P < 0.05$) from dexmedetomidine-alone measurement.

behavioral response to a drug has relied heavily on the use of pharmacologic probes. Unfortunately, in the field of α_2-adrenoceptor pharmacology this has proven to be unhelpful because of the lack of subtype-selective ligands. The only compound that has proven to be at all useful is the prototype α_1 antagonist prazosin, which has between a 10- and 100-fold higher affinity for the α_{2B} and α_{2C} subtype than for the α_{2A} subtype, respectively.[33,34] Yohimbine is used to distinguish pharmacologic actions at the α_2 adrenoceptor (which it blocks) from the α_1 (at which it has extremely low affinity) adrenoceptors. Because yohimbine is an effective blocker of the response (fig. 4), it is clear that the antinociceptive response to N_2O is cause by α_2 and not α_1 adrenoceptors. Therefore, the attenuation by prazosin of the antinociceptive action of N_2O is caused by its activity at $\alpha_{2B/C}$ adrenoceptors and not by its well-described ability to block α_1 adrenoceptors.

Gene targeting has provided a novel approach to study the functional role of identified proteins in response to hormones, neurotransmitters, and pharmacons (for review see reference [35]). In studies related to anesthesia, Matthes *et al.*[36] demonstrated that the μ-opioid receptor gene product is the molecular target of morphine *in vivo*. Addressing the α_2-adrenergic receptor, we reported that the entire repertoire of anesthetic and analgesic actions of dexmedetomidine, the α_2 agonist, was blocked in D79N mice.[27] The hypnotic response to barbiturates, the anesthetic-sparing action of adenosine, and the antinociceptive response to morphine were each unaltered in the D79N mice, demonstrating the specificity of the altered molecular component. Actions mediated by the α_{2B}-receptor subtype, including hypertension,[29] remain unaffected in mice with the dysfunctional α_{2A}-receptor subtype.[28] Therefore, D79N transgenic mice have a discrete and selective defect only in the signaling pathway involving the α_{2A}-receptor subtype. Although the data from the studies cited are relatively unambiguous, there are caveats that need to be addressed. It is possible that other genetic loci in the strain of mice from which the embryonic stem cells were derived can account for some of the changes. This can be addressed by back-crossing through multiple generations to yield a congenic strain that differs in only one genetic locus from its wild type. Also, the genetic alteration may induce a compensatory change that could affect the behavioral phenotype. This can be resolved by performing "rescue" experiments in which the genetically altered mice are mated with a strain that overexpresses the original gene, thereby diluting the contribution of the altered gene.

One issue that requires resolution is the fact that an exogenously administered α_2 agonist exhibits no analgesic response in D79N mice, whereas the N_2O-provoked release of an endogenous α_2 agonist still produces analgesia in D79N, albeit less pronounced than that found in the litter mates. Thus, N_2O does not depend, exclusively, on the α_{2A} adrenoceptor to transduce its antinociceptive response, as is the case for the exogenously administered α_2 agonist (fig. 7A); rather, N_2O can use non-α_{2A}-adrenoceptor subtypes. The reason for this difference

between endogenously and exogenously generated α_2 signaling is unlikely to be caused by the agonists themselves, because dexmedetomidine (the exogenous agonist) and norepinephrine (the putative endogenous agonist) both are relatively nonselective with respect to the α_2-adrenoceptor subtypes. In our previous report with dexmedetomidine, we used a ramping hot-plate nociceptive paradigm rather than the TFL response in which only a spinal reflexive nociceptive pathway is involved.[37] However, we subsequently showed that dexmedetomidine has no antinociceptive properties in the D79N when the TFL paradigm was used (fig. 7A). Another pertinent difference between the two studies is the fact that dexmedetomidine was administered systemically whereas, during N_2O exposure, norepinephrine was presumably released at the terminals of the descending noradrenergic pathway in the dorsal horn of the spinal cord. However, it has been shown recently that even intrathecally administered dexmedetomidine is ineffective in the tail-flick test in D79N mice.[38] The current findings are consistent with our previous observation that systemic morphine is as effective in the D79N as in the wild-type mice, because previous studies showed that morphine works, in part, through the supraspinal activation in the periaqueductal gray of a descending noradrenergic pathway, which is presumably similar to the action of N_2O.

The results from our study have potentially far-reaching consequences because they illustrate that the α_{2B}- or α_{2C}-receptor subtypes, or both, are capable of mediating an analgesic response. These proteins represent a potential target for subsequent novel drug design and synthesis for analgesia and provide the hope that the sedative effects of α_2 agonists may be avoided because we showed that this property is transduced exclusively at the α_{2A}-receptor subtype.[27,39]

In conclusion, we showed that N_2O antinociception involves opiate and α_2-adrenergic receptor transduction pathways in mice, which represent an important species in which to further investigate the molecular mechanism because of its accessibility to gene transfer technology. In a transgenic mice strain with dysfunctional α_{2A} adrenoceptors, we are still able to demonstrate a significant antinociceptive response to N_2O, indicating that α_{2B} and α_{2C} subtypes also participate in this response. Studies are ongoing to develop mice lacking α_{2B} or α_{2C} subtypes, or both, to determine whether their involvement is sufficient to produce the antinociceptive response to N_2O.

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