HISTORY

Nitrous oxide (N2O), or laughing gas, has had an intriguing history from its discovery to modern clinical application. Although John Mayow of 17th century is recognized as the first person who isolated "nitrous air" (a mixture of nitric oxide, nitrogen dioxide and nitrous oxide), Joseph Priestly of 18th century in England is usually regarded as the discoverer of N2O. Sir Humphry Davy of England first reported the analgesic effect of N2O in 1800, and Horace Wells of the United States in the 19th century has been credited as the first clinician who successfully applied its analgesic properties to surgical operations.

CLINICAL UTILITY

Administration of N2O to humans and animals causes relatively potent analgesic/antinociceptive effects and weak anesthetic/hypnotic effects. These effects are not strong enough for N2O to be used by itself for surgical anesthesia except for minor operations such as dental procedures. Nevertheless, N2O is often used for general anesthesia in combination with other drugs, because the addition of N2O reduces the requirement of other analgesic and anesthetic agents. In addition, N2O possesses sympathomimetic effects, which are beneficial to counteract the sympatholytic effects of the volatile anesthetic agents, e.g., isoflurane, sevoflurane and halothane, which are usually co-administered with N2O. Other benefits of using N2O with another volatile anesthetic agent stem from its physical characteristics, i.e., low solubility.

PREVIOUSLY ESTABLISHED MECHANISMS:

While the anesthetic/hypnotic mechanisms of N2O remain largely unknown, the underlying mechanisms of its analgesic/antinociceptive effects have been rapidly elucidated during the past several decades. Both supraspinal opiate and spinal adrenergic receptors are involved in the antinociceptive response (which is analogous to an analgesic response in non-communicative animals) to N2O. A descending inhibitory pathway connects the initiating mechanisms in the brain to the modulating mechanisms within the spinal cord. Over the course of the last 5 years we have elucidated the molecular and neural substrates involved in the analgesic action of N2O at both the supraspinal and spinal levels, and have explored how these new insights may influence patient care.

EVIDENCE FOR THE SUPRASPINAL MECHANISMS INVOLVED IN THE ANALGESIC ACTION OF N2O

Figure 1 shows a schematic representation of our principal published findings detailing the supraspinal effects of N2O.

Using a combination of behavioural, pharmacological and immunohistochemical techniques we investigated the role of Corticotrophin Releasing Factor (CRF)-containing neurones for the action of N2O [1]. Brain sections including the hypothalamus were immunostained for both c-Fos (a marker of neuronal activation) and CRF, and the percentage of CRF-positive neurones expressing c-Fos was determined. The functional consequences of changes in CRF-containing neurones were investigated by assessing the effect of intracerebroventricular administration of a CRF antagonist (alpha-helical CRF9-41) on both activation of locus coeruleus noradrenergic neurones and the analgesia produced by nitrous oxide. Inhalation of nitrous oxide induced a concentration-dependent increase in c-Fos expression in CRF-positive neurones in the paraventricular nucleus of the hypothalamus. Intracerebroventricular administration of the CRF antagonist inhibited nitrous oxide-induced c-Fos expression in the locus coeruleus and ablated the antinociceptive effect of nitrous oxide. We interpreted these data to indicate that N2O activates the CRF system in the brain, thereby provoking the release of endogenous opiate ligands which, we have previously demonstrated, produce activation of opiate receptors in the periaqueductal grey region of the mid-brain.
Effects of Nitrous Oxide ($N_2O$) on Supraspinal Neuronal Pathways Activity in a putative neural network (extending from the hypothalamic regions where corticotrophin releasing factor [CRF] containing neurones originate to the cell bodies and spinal projections of noradrenergic neurones in the ponsmedulla) is shown in the absence (“resting state”) and presence of $N_2O$. Activation is depicted by a filled circle in the cell body. Manuscript citations of the principal findings are listed.

Because activation of the opiate receptors in this mid-brain region is associated with an increase in activity in the locus coeruleus (LC), we proposed that the opiate receptors in the mid-brain are located on a neuronal pathway which, when active, will inhibit the noradrenergic neurones in the LC; thus, when the endogenous opiate ligands bind to the opiate receptor, this pathway is *inactivated*, thereby *disinhibiting* the activity within the LC. Evidence that the noradrenergic pathway is under inhibitory control of a GABAergic pathway is supported by our finding that microinjection of the GABA$_A$ agonist, muscimol, into the brain stem inhibited $N_2O$-induced c-Fos expression in the pontine noradrenergic nuclei and ablated its analgesic effect. Thus we propose that the descending noradrenergic pathways are tonically inhibited by GABAergic neurones; loss of this inhibition (through inhibition of the GABAergic interneurones) activates the descending noradrenergic pathways which modulates pain processing in the spinal cord. We postulate that opioid peptide release leads to inhibition of GABAergic neurones *via* activation of opiate receptors [2].
We sought to determine whether activation of the noradrenergic neurones in the pons-medulla is required for analgesic action of N₂O. After application of the mitochondrial toxin saporin, coupled to an antibody directed against dopamine β hydroxylase (DBH-saporin) nearly all of the catecholamine-containing neurones in the pons were eliminated; this lesion blocked the analgesic effects of N₂O [3].

**Evidence for the Spinal Mechanisms Involved in the Analgesic Action of N₂O**

Turning our attention to events in the spinal cord (Figure 2), we investigated whether the activation of the noradrenergic nuclei in the brainstem resulted in a release of norepinephrine in the dorsal horn of the spinal cord using in vivo microdialysis [4]. After exposure to N₂O, there was a fourfold increase in norepinephrine which was blocked by naltrexone. Depletion of norepinephrine in the spinal cord blocked the analgesic response to N₂O. These finding establish that a descending noradrenergic pathway in the spinal cord links N₂O-induced activation of opiate receptors in the periaqueductal grey, with activation of adrenoceptors in the spinal cord by norepinephrine.

**Figure 2:**

Effects of Nitrous Oxide (N₂O) on Spinal Neuronal Pathways Activity in a putative neural network (extending from the spinal projections of noradrenergic neurones in the pons-medulla to the components of nociceptive trafficking in the dorsal horn of the spinal cord) is shown (the terminal of the GABAergic interneurone on the primary afferent neurone has not been established). Activation is depicted by a filled circle in the cell body. Manuscript citations of the principal findings are listed.

In order to determine the types of adrenoceptors that are activated by norepinephrine, we studied the effect of pharmacological probes and genetically modified animals on the analgesic response to N₂O. Prazosin, an α adrenoceptor antagonist with affinity for both the α₀₂B and α₁ subtypes, blocked the analgesic action of N₂O [5]. Using knockout animals that lacked either the α₀₂A, α₀₂B, or α₀₂C subtypes we showed that the analgesic action of N₂O was blocked only when the α₀₂B subtype was absent [3]. These findings do not exclude the possible participation of α₁ adrenoceptors as well; therefore, we explored whether this receptor subtype was responsible for the activation by N₂O of neurones in the dorsal horn of the spinal cord which we reported earlier [12]. We established that N₂O-induced c-Fos expression was co-localized with α₁ adrenoceptor immunoreactivity in laminae III-IV [6].
We investigated the types of neurone that were activated by N₂O in the dorsal horn of the spinal cord [12]. Induction of c-Fos by N₂O was restricted to cells that stained for the presence of glutamic acid decarboxylase, the rate-limiting enzyme involved in the synthesis of GABA; thus, exposure to nitrous oxide activates GABAergic neurones in the spinal cord [7].

**Exploring Clinical Extrapolations**

(i) Because functional communication between the supraspinal and spinal sites is essential to express the analgesic properties of N₂O, we wondered whether N₂O would be efficacious in developmentally-immature rats in whom connections are not yet formed.³ We established that neither the analgesic action of N₂O to thermal stimuli [8] nor c-fos activation by N₂O was present in rats below three weeks of age [9]. In order to resolve possible confounding variables associated with behavioural studies in neonatal rats involving thermally-induced pain, we conducted a series of studies in which pain was provoked by formalin-injection into the hindpaw. This allowed us to assess the putative analgesic action of N₂O in response to an inflammatory stimulus, and to corroborate the behavioural assessments with an immunohistochemical study to determine whether N₂O could modulate pain processing in the spinal cord by its ability to suppress formalin-induced c-Fos expression [10]. Adult-like antinociceptive responses to N₂O, both behaviourally and immunohistochemically, were only present in rats older than 3 weeks (23- and 29-day-old). If extrapolatable to humans, this suggests that N₂O would be ineffective as an analgesic in humans below the toddler stage, because a three-week old rat is developmentally equivalent to a human of 2 years.³

(ii) Drugs that enhance GABA<sub>A</sub> receptor function (“GABAergic agents”), including the volatile anaesthetics⁴, intravenous anaesthetics⁴, and benzodiazepines⁵ are frequently used in the perioperative setting. From our studies addressing the mechanisms for the analgesic action of N₂O, we have invoked both a supraspinal inhibition [2] as well as a spinal activation [7] of GABAergic neurones. To dissect how the analgesic efficacy of N₂O would be affected by GABAergic agents, adult rats were discretely administered muscimol (a GABA<sub>A</sub> receptor agonist), supraspinally, or gabazine (a GABA<sub>A</sub> receptor antagonist), spinally, prior to exposure to N₂O. In support of our proposed mechanism for the analgesic action of N₂O, when supraspinal inhibition or spinal activation of GABAergic neurones are overcome, the analgesic action of N₂O, assessed both behaviourally and immunohistochemically, is blocked; a priori it was not possible to speculate which of these two actions would predominate when GABAergic agents are used. Systemically-administered midazolam inhibited N₂O-induced activation of the descending noradrenergic pathway and thereby its analgesic action. Thus, even though systemically-administered midazolam can modulate GABA<sub>A</sub> receptors both supraspinally and spinally, the dominant action is at the supraspinal GABAergic site producing a pronociceptive action.

(iii) To establish the most appropriate rat strain for studies of analgesic actions of N₂O, we examined efficacy and durability of responsiveness in two outbred and four inbred strains. In the outbred strains (Sprague-Dawley from two different breeders) the peak response occurred after 30 min of exposure, and tolerance to N₂O developed within 60 to 90 min. Each of the four inbred strains examined, i.e., Wistar-Kyoto, Brown-Norway, Fischer, and Lewis, displayed a unique pattern of antinociceptive response to N₂O. Wistar-Kyoto and Brown-Norway strains showed somewhat similar patterns as those observed in outbred strains, apart from the fact that the Wistar-Kyoto displayed a more distinct development of tolerance, whereas the Brown-Norway strain had a lower peak effect. The Fischer strain displayed the greatest antinociceptive response to N₂O, and did not develop tolerance. The Lewis strain showed no antinociceptive response to N₂O. When the strain differences in the genes expressing the molecular components involved in the analgesic action to N₂O are revealed, we can exploit this information in pharmacogenomic studies in humans by exploring N₂O phenotypes and correlating these with single nucleotide polymorphisms.
REFERENCES