Laughing gas: no laughing matter

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Abstract

Nitrous oxide, also known as laughing gas, is a gas often used as an anaesthetic and analgesic agent. More recently, nitrous oxide is being used for recreational purposes as it induces a “high” feeling. The Dutch government is planning to ban the recreational use of nitrous oxide based on problems like traffic accidents, littering and risks for public health. However, not much is known about the biological effects of nitrous oxide on the human body. This essay will try to investigate if nitrous oxide should be prohibited based on its biological effects. The increase in popularity of this gas amongst teens and students is followed by a rise of neurological and sensory perceptive complaints. Nitrous oxide has a quick onset and efficient clearance due to its low blood/gas coefficient. The gas is not very potent and its mechanism of action is not completely understood. The opioid hypothesis speculates that it stimulates the release of opioid peptides in the periaqueductal gray matter (PAG) which indirectly leads to a lower pain perception. Other papers propose that the gas acts as an NMDA glutamate receptor antagonist. Finally research shows that nitrous oxide causes a cobalamin (vitamin B_{12}) deficiency. The effect of the gas on brain activity remains unclear, but seems to alter slow-delta oscillations. On a molecular level, the gas seems to induce cell proliferation in the hippocampus and protect the brain against NMDA induced neurodegeneration. However, when the concentration of nitrous oxide becomes too high, neurotoxicity can be observed in the posterior cingulate/retrosplenial (PC/RS) cortex. When the gas is combined with ketamine, this neurotoxicity in increased. Because nitrous oxide seems to enhance dopamine levels in the nucleus accumbens (NAc), the final chapter of this essay looks at the addictive potential of nitrous oxide where various aspects of addictive substances are evaluated. Because the duration of exposure and the dose of nitrous oxide are important factors when considering the biological effects, it does not seem necessary to ban the use of this drug. Instead, informing users about the risks just like with alcohol and tobacco, might be a more effective solution.

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Abbreviations

% MRC:	Percentage of maximum control response
AN:	Arcuate nucleus
AR:	Adrenoreceptor
Atm:	Atmospheric pressure
CAM:	Coordination Point Assessment and Monitoring new drugs
CNS:	Central nervous system
CPP paradigm:	Conditioned place preference paradigm
CRF:	Corticotropin-releasing factor
EEG:	Electroencephalography
EtOH:	Ethanol
ExNT:	Excitatory neurotransmitters
GABA:	Gamma-aminobutyric acid
HAD rats:	High alcohol drinking rats
HCY:	Homocysteine
ICSS:	Intracranial self-stimulation
LVA:	Low voltage activated
MAC:	Minimum alveolar concentration
MLDS:	Mesolimbic dopamine system
MMA:	Methylmalonic acid
MMCoAM:	Methylmalonyl CoA mutase
MS:	Methionine synthase
N_2O:	Nitrous oxide
NAc:	Nucleus accumbens
NE:	Norepinephrine
NMA:	N-methyl-D, L aspartic acid
NMDA:	N-methyl-D-aspartate receptor
NO:	Nitric oxide
NVWA:	Nederlandse Voedsel- en Warenautoriteit
OP:	Opioid peptides
P rats:	Alcohol-preferring rats
PAG:	Periaqueductal gray matter
PC/RS cortex:	Posterior cingulate/retrosplenial cortex
RIVM:	Rijksinstituut voor Volksgezondheid en Milieu
THF:	Tetrahydrofolate
TREK-1 channel:	Two-pore-domain potassium channel
VTA:	Ventral tegmental area

Introduction

On October 4th 2021, a Dutch newspaper reported a car crash during which two people got hurt and were brought to the hospital. It seems like an ordinary article since (sadly) car crashes happen every day. However, after the crash the motorway was covered with over 60 cylinders of nitrous oxide, also known as laughing gas (**Figure 1**).[1] This is not the first time such an article is published as it seems more people are using laughing gas while participating in traffic. Just between January and July of 2019 there were a total of 960 traffic incidents as a result of laughing gas consumption which is a lot more compared to the 380 incidents during 2018.[2]

Figure 1: A) Car crash on the Dutch highway as a result of the use of laughing gas. B) Cylinders of laughing gas found at the location of the crash. Van der Put, T. Ongeval op A16 bij Breda [Online image] BN de Stem. https://www.bndestem.nl/breda/meerdere-auto-s-botsen-op-a16-bij-breda-snelweg-bezaaid-met-flessen-lachgas~a2c25eba/

Nitrous oxide or laughing gas, is a colourless, non-irritating, slightly sweet smelling gas that is also known as nitrous, nos or N_2O. The gas is often used as an anaesthetic agent in the hospital or as a temporary analgesic agent by dentists and ambulance personnel as it inhibits the sensation of pain and has a calming effect. Next to these anaesthetic and analgesic effects, it also inhibits bacterial growth, making it an appropriate aerosol spray propellant mostly used for whipped cream cans and cooking spray. Moreover, nitrous oxide supports combustion, a process which is used for car- and motorsports as well as for rocket engines.[3] Besides these practical applications, it is used as a drug to induce a "high" feeling. It is often inhaled via balloons which are filled from a tank, cylinder or whipped cream bulbs called "whippets" to induce an euphoric effect for a short amount of time.[4] The number of balloons or hours of exposure varies greatly among users with extreme cases using 100-200 bulbs per day or exposure up to 6 hours per day for over a month.[5] [6]

The Dutch National Institute of Public Health and the Environment (RIVM) reported that the use of 5-10 balloons per session with a maximum of once a month could not induce detrimental effects on your health. However, the Dutch Coordination Point Assessment and Monitoring new drugs (CAM) stated in a report from 2019 that the inhalation of the gas can be a risk for public health. Therefore, they advised the government to discourage the recreational use of nitrous oxide.[7] [8] The CAM is an organisation which includes members of the Healthcare Inspectorate, Dutch Food Safety Authority (NVWA) and the Trimbos-institute created with the goal to perform a risk assessment when drugs newly appear in society.[9] In summary this CAM report states that the risks of nitrous oxide on the health of an *individual* are low to moderate, the acute health effects are limited and it is difficult to set a usage limit when recreational use of nitrous oxide appears to be safe. A third of the users who uses between 5-10 balloons, shows acute unwanted side effects like headache, dizziness and tingling in the hands and feet. The risk for *public health* is considered moderate to large but the chance of disturbing public order is low to moderate as nitrous oxide does not induce aggressive behaviour. However, littering of balloons, whippets and cylinders is a big societal burden. The risks for accidents when using nitrous oxide in traffic are large due to a decreased responsiveness and the distraction of blowing up a balloon while driving. Because the gas can easily and legally be obtained, the risk of involvement in criminal organisation is small. Overall nitrous oxide scores high in this risk assessment, which is why the CAM advised the government to discourage the recreational use of the gas and limit the distribution.[8]

In December of 2019 the Dutch government announced a plan to ban the recreational use of nitrous oxide starting 2021 as a result of this report.[10] At the moment nitrous oxide is placed under the Dutch Commodities Act (*Warenwet*) which makes it legal to possess and use.[10] This causes the gas to be widely available in the Netherlands. The gas could be banned by placing it under the Opium Law which is a section of Dutch law that covers psychotropic drugs. More specifically it would be placed on *List II* of this Opium Law, which is the list for soft drugs. Users of these listed soft drugs cannot be prosecuted but possessing, producing or trafficking of these substances is a criminal offence.[11]

The traffic accidents, littering and public health risk of nitrous oxide are reasons for this ban. However, not much is known about the biological effects of this gas. This essay will try to investigate if the recreational use of nitrous oxide should be prohibited based on its biological effects. To fully understand these effects, this essay will first focus on the history and application of nitrous oxide in society. The following chapter will go into the characteristics of the gas followed by a chapter explaining its mechanism known so far. In addition some papers on the effects of nitrous oxide on the brain will be discussed. Its addictive potential for abuse will be investigated based on animal and human experiments in the final paper followed by a discussion.

Chapter 1: History and application

Joseph Priestley was the first person reported to initially synthesize nitrous oxide in 1772. Around 1785, Humphrey Davy became known for his work on the synthesis and inhalation of small amounts of nitrous oxide.[12] Davy was the first person to report euphoric and relaxant properties after inhalation.[13 14] The produced gas was captured and used to entertain people with its euphoric and dizzying effects and this ability gave the gas its name "laughing gas". People even inhaled it during demonstrations at exhibitions and shows.[12] So the recreational use of nitrous oxide is not something new as it dates back to Victorian times when "laughing gas parties" were popular (**Figure 2A**).[15]

Figure 2: A) Laughing gas parties in Victorian times. B) Early administration of nitrous oxide during tooth extraction.
Drawing A: https://www.ancient-origins.net/history-important-events/laughing-gas-parties-0011605
Drawing B: Edmond EI II, Philip LC. Anesthetic solubility in blood and tissues: values and significance. Br J Anesth. 1964;36:140–9

Later, Gardner Quincy Colton learnt the effects of nitrous oxide during his medical studies and quit his studies to give lectures about the drug. One of his audience members was dentist Horace Wells, who noticed that a volunteer was not aware of his injury after hurting himself following inhalation of nitrous oxide.[3 16] Wells established the use of nitrous oxide in dentistry in order to reduce pain and Colton began using 100% nitrous oxide during tooth extraction (**Figure 2B**). Around 1868, Andrews proposed to add 20% oxygen to nitrous oxide to create a safer and more pleasant anaesthetic gas.[17] It has been known for quite a long time that the use of nitrous oxide is paired with some risks. Even after the second world war there were still some practitioners who administered pure (100%) nitrous oxide, also known as "blue gassing", which caused a deficiency in oxygen supply to the blood, known as hypoxia. In 1939, Courville published a book to describe the harmful effects of hypoxia as a consequence of nitrous oxide inhalation, even with 20% oxygen. Because of this hypoxia and because administration of nitrous oxide could only last a short amount of time, this led to the development of new techniques with lower concentrations of nitrous oxide.[12 17]

Nowadays, nitrous oxide is often used as an anaesthetic agent to induce an anaesthetic state. This is a state which is characterized by lack of perception of all sensations with or without losing consciousness. General anaesthesia is often needed to perform surgery, when a patient needs to be immobile (skeletal muscle relaxation), unconscious and when excessive autonomic response to surgical stimulations should be attenuated. Inhalation agents often come closest to accomplishing all three factors for general aesthetics, but only at such high doses that brain function is limited, which can endanger vital functions.[18] Because not many general anaesthetics agents live up to all three expectations, it is common practice to combine treatments. In such a combined treatment, inhalation agents like nitrous oxide often cause unconsciousness, while opioids induce analgesia and neuromuscular blocking agents induce muscle relaxation.[17 18] Nitrous oxide does not reduce net ventilation, mean arterial pressure or provides skeletal muscle relaxation unlike other inhalation anaesthetics. This is why nitrous oxide is generally considered a harmless, slow, passive gas with little influence on vital physiological functions.[18] However, with more and newer anaesthetic agent available, nitrous oxide is has become less popular as an anaesthetic agent.

Because nitrous oxide on its own is not potent enough as a sole anaesthetic, which is needed for a surgery, it can be really useful in situations where full anaesthetic is not desirable. That is why subanaesthetic concentrations of nitrous oxide are often used in dentistry as a drug to reduce anxiety (anxiolytic) and to produce analgesic effects. This means that there is pain relief without losing consciousness.[3] It is even argued that nitrous oxide is the safest available method to induce sedation in dentistry.[18] It is used by first responders who are not trained to administer intravenous analgesics.[19] Nitrous oxide is placed on the World Health Organization's list of Essential Medicine, which lists the safest and most effective medicines in health-care system.[20]

Next to these medical purposes, the drug has gained popularity as a recreational agent due to its low cost and easy availability.[12] [17] Approximately two decades ago, nitrous oxide became popular again as an inhalant drug, also named "Hippy Crack", especially in clubs and music festivals.[21] When the gas is inhaled it causes depersonalization, derealization, psychotomimetic effects, dizziness, excitement, heightened consciousness, and euphoria.[3] [17] It has a very rapid onset of action and it peaks at 1 minute after inhaling the gas. The effect also disappears quickly, after 2 minutes with no after effects, making it possible to resume activities immediately after using it.[17] The recreational use of "laughing gas" has increased even more over the past few years, especially among teens and students. In 2018 the Trimbos-institute conducted a survey among 16 to 18 year olds about their drug use. They reported that almost one out of three (30%) of these students had ever used laughing gas in their lifetime. Out of this 30%, 8% had used laughing gas the month just prior to the survey with 23% using it more then 5 times during this month. When comparing this data to previous years, there is an increase visible in the number of laughing gas users in this age category. In 2015 only 20% had ever used laughing gas with 5.2% having used it in the month prior to the survey.[22] Laughing gas usage was also reported at an even younger age of 12 to 16 year olds. In the year 2019 one out of 10 (10%) of students had ever used laughing gas in their lifetime. Most of these students used one (42%) or between 2-4 (36%) balloons per session. However, some students used between 5-9 balloons (12%) or even 10 or more balloons (10%) per session. The more recent users also showed increase in laughing gas use per session, with 20% using between 5-9 balloons and 20% using 10 or more balloons. In 2015 only 8% of 12 to 16 year olds had ever used laughing gas.[7]

This increase in popularity was also visible in the Dutch nightlife as nitrous oxide filled balloons became available in clubs and pubs in the Netherlands. To limit public disturbance, signs were placed at the beginning of nightlife areas to prohibit the use of the drug in the street (**Figure 3**). As the recreational use of nitrous oxide increases in popularity, more people report to the hospital with neurological complaints (myelopathy, myeloneuropathy) and disturbances in sensory perception like tingling or no feeling in the hands. Even deafness, mood changes, psychosis and motor weakness are observed.[3] [23] This increasing number of nitrous oxide users together with these reported complications is reason for concern.

Figure 3: Sign at the beginning of a street in Groningen, The Netherlands meaning "Prohibited to use laughing gas".

In summary, nitrous oxide is not a new drug as it was initially synthesised 250 years ago and people have continued to use it throughout the years since, for both medical and non-medical purposes. Soon after its discovery it has been optimised as an anaesthetic and analgesic agent and it is considered a harmless gas when used in low concentrations. Despite the fact that it is being used as a recreational agent for a longer time, there is an recent increase in popularity especially amongst teens and students. With this increase in popularity, more neurological complications are being reported raising concern. Because there is not much literature available on the recreational use of nitrous oxide, the main focus of the following chapters will be on literature describing the effects of nitrous oxide when used as an anaesthetic or analgesic agent to determine its biological effects.

Chapter 2: Characteristics

As mentioned earlier, nitrous oxide is a sweet smelling, non irritating, colourless gas with the formula N_2O. It is not inflammable but at temperatures above 450 °C it breaks down into nitrogen and oxygen which later supports combustion. It is a stable gas and chemically inactive at room temperature.[17] Nitrous oxide is industrially produced by thermal decomposition of ammonium nitrate by heating a solution of ammonium nitrate and water to approximately 250 °C. At this temperature the ammonium nitrate decomposes into nitrous oxide and impurities are washed out. Nitrous oxide becomes liquid under the applied pressure and is stored in tanks, cylinders and bulbs or so called "whippets".[12] The bulbs or "whippets" normally used to fill balloons, contain 10 ml of nitrous oxide in liquid form under pressure, which is 4 litre of gas under normal conditions. It is inexpensive as it only costs between 5-8 euro's for 24 bulbs.[21]

Background 1: Partial pressure & Partition coefficient

When there is a mixture of gasses, each separate gas contributes to the total pressure of this mixture with its own **partial pressure**. Adding all partial pressures up, will give the total pressure of a gas mixture. Partial pressure is important for the equilibration of tensions from inspired gas, alveoli and arterial blood. Gas exchange from air (alveoli) into a liquid (blood) is proportional to the pressure gradient of the gas and the solubility of the gas. Gas molecules dissolve from air into blood when the partial pressure is higher in the alveoli compared to capillaries because gas moves from a higher to a lower partial pressure. [54 57] This diffusion will continue until an equilibrium is reached, meaning that the partial pressure of the inspired anaesthetic gas in the alveoli is equal to the partial pressure of the anaesthetic agent in blood or tissue. This equilibrium in partial pressure does not mean that concentration of anaesthetic agent is equal in air and blood. The concentration of dissolved anaesthetic agent also depends on the solubility of the agent in blood. [57 58]

A **partition coefficient** can be used to express how soluble inhaled anaesthetics are in different types of solvents.[54] The **blood/gas partition coefficient** indicates the solubility of an inhaled gas in blood as it describes the ratio of the drug in blood and in inhaled air at equilibrium of partial pressures.[55 56]

- A higher blood/gas partition coefficient means that the inhaled anaesthetic is more soluble in blood in comparison to air. This causes a slow onset and more anaesthetic gas is needed to induce the desired effect (**Figure 4A**).
- On the other hand, if the blood/gas partition coefficient is lower, the inhaled anaesthetic is less soluble in blood in comparison to air. This causes a quick onset and less anaesthetic gas is needed to induce the desired effect (**Figure 4B**).[54 55 56]

Effects of solubility on the onset of gas anaesthetics

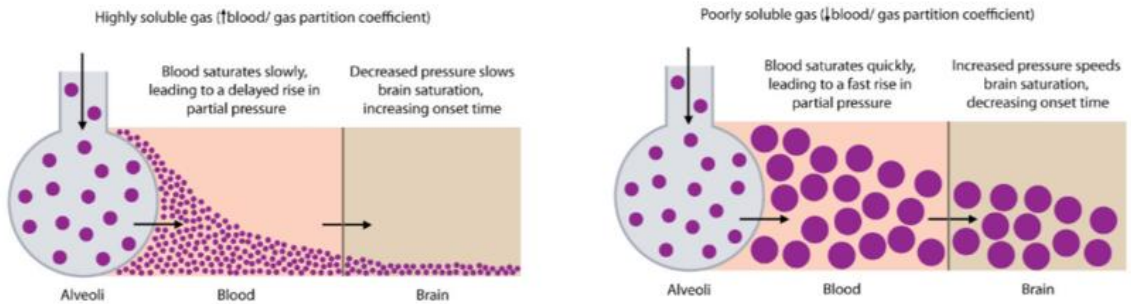

Figure 4: A) Highly soluble gas has a high blood/gas partition coefficient which leads to slower rise in partial pressure in the capillaries and thus a slower onset of drug action. B) Poorly soluble gas has a low blood/gas partition coefficient which leads to a faster rise in partial pressure in the capillaries and thus a faster onset of drug action. [54 55 56]

Nitrous oxide has a low blood/gas partition coefficient of 0.47, meaning that it has a low solubility in blood (**Background 1**). This means that only a small amount of nitrous oxide is absorbed by blood which causes nitrous oxide molecules to be quickly transported to the brain. This works as follows, nitrous oxide fills the alveoli as it enters the lungs and these alveoli are surrounded by a network of pulmonary capillaries. The pulmonary capillaries have no nitrous oxide yet when the anaesthetic gas enters the alveoli. Due to the difference in partial

pressure between the air in the alveoli and blood in the capillaries, the anaesthetic gas will diffuse from the alveoli into capillaries. The alveolar concentration of nitrous oxide is the result of a balance between the inhaled nitrous oxide into the lungs and the removed nitrous oxide taken up by the blood. If no gas would diffuse into the blood, then the alveolar concentration would rise until it equals the amount of inhaled gas. Due to the low solubility of nitrous oxide, only a small amount of nitrous oxide is quickly absorbed into the blood after the gas has entered the alveoli, causing the alveolar concentration to rise rapidly towards the inhaled concentration. This high alveolar concentration causes a rapid transfer of nitrous oxide molecules. The small amount of nitrous oxide which has diffused into the blood in the capillaries, reaches vessel rich groups like the brain, the heart and the kidneys. Again, because of the low solubility of nitrous oxide, a rapid equilibration is formed between the blood and these tissues.[24] Blood returning to the heart, after equilibration with these tissues, cannot take up any more nitrous oxide. This causes the alveolar concentration to increase even further and nitrous oxide can quickly equilibrate in less perfused tissue, like fat and muscles.[17] The rapid equilibration between the different partial pressures of the inspired gas, alveoli and arterial blood causes nitrous oxide to be rapidly taken up in the blood and together with the quick transport of nitrous oxide to the brain it causes a quick onset of effects. [17 18 24] The decreased solubility of nitrous oxide also means a faster recovery from the gas, because tissues have a low nitrous oxide saturation and there is less reservoir for the gas. There are also no metabolic pathways for nitrous oxide.[24] This causes an efficient and effective clearance of nitrous oxide with a minimum recovery time as a result.[17]

As mentioned in the previous chapter, pure nitrous oxide gas should not be inhaled continuously for a long time since the body needs oxygen to survive, which means that these gas mixtures always contain oxygen. Nitrous oxide has a MAC of 1.04 or 104%, making it the least potent of anaesthetic gases (**Background 2**). Its potency is so low that is not even possible to administer nitrous oxide concentrations higher than 75% under normal pressure at sea level without altering oxygen supply, which must be kept at 25%. This is the reason why the concentration of nitrous oxide is never higher than 75% of the inhaled anaesthetic mixture in human anaesthesia. Because this 75% nitrous oxide is not enough to induce full anaesthesia it is often combined with other anaesthetic drugs.[25] This low potency is also the reason why nitrous oxide can only be studied under hyperbaric conditions, when a higher pressure increases the density of gas molecules.[17] Finally it is important to note that many variables and additional drugs can change the MAC of nitrous oxide in a given patient. For example depressants of the central nervous system like intravenous sedatives or opioid can change the MAC of an inhaled anaesthetic. Factors that increase the MAC include fever, central nervous system (CNS) stimulants, young age and chronic alcoholism. Factors that decrease MAC on the other hand include CNS depressants, increasing age and hypothermia.[18] When using animal studies to investigate the effects of nitrous oxide, it should be taken into consideration that different concentrations of nitrous oxide in rodents and humans have a relatively different effect sizes. For example the MAC of nitrous oxide used in rats is 155% while in humans this is 104%.[25 26]

In summary, nitrous oxide has a low blood/gas coefficient, meaning that it has a quickly reaches the brain and has a rapid onset. This also causes an effective and efficient clearance of the drug with a short recovery time. The MAC of nitrous oxide is low, which indicates that it is not very potent, making it difficult to administer high concentrations of nitrous oxide without using hyperbaric conditions. When reading the following chapters, it should be kept in mind that the percentages of nitrous oxide used in animal studies cannot directly be translated to concentrations needed in human subjects.

Background 2: Minimum alveolar concentration (MAC)

As mentioned before, nitrous oxide is often combined with other inhalation agents to induce anaesthesia. The dose of the anaesthetic gas in an anaesthetic mixture can be expressed as a percentage of the inspired mixture. The minimum alveolar concentration, also known as MAC, is used as a measure for the potency of a single inhaled gas. It represents the concentration of an anaesthetic gas in the alveolus (at 1 atmosphere) that renders 50% of patients unresponsive to a surgical stimulus. In other words, it indicates the percentage of a gas required to provide anaesthesia in 50% of patients. This potency measure makes it possible to compare the potencies of different anaesthetic gases. Because MAC indicates the sufficient dose for unresponsiveness in 50% of patients, other patients might need between 0.5 to 2.0 MAC to achieve successful anaesthesia. The average MAC needed to render 90% of patients unresponsive to surgical stimulus is 1.3 MAC and a maximum of 1.5 to 2.0 MAC is needed to induce anaesthesia in all patients.[18] These MAC values are useful to determine what the proportion of an anaesthetic in an inhaled anaesthetic mixture should be, depending on its desired effect. For example nitrous oxide is often used to reduce the MAC of an additional inhalation anaesthetic agent.[17]

Chapter 3: Mechanism of action

Nitrous oxide has been clinically used for over 150 years but its complete mechanism of action remains unclear. It is important to realize that the concentration of nitrous oxide determines its effects. When a high anaesthetic concentration is used, nitrous oxide causes unconsciousness. However, at a lower concentration, consciousness is preserved and analgesia, anxiolysis and euphoria are induced.[17] Next to these effects, nitrous oxide also causes side effects via the oxidation of cobalamin, also known as vitamin B_{12}. This chapter will first elaborate on the perception of pain before going into the above mentioned effects.

When a human experiences physical pain, a pain signal transfers from the place of injury via ascending tracts to the sensory cortex of the brain. This signal passes through the spinal cord via nerve fibers to the medulla, pons and midbrain of the brainstem (**Figure 5, red neurons**). More precisely, during pain sensation, sensory nerve fibers or so called first-order neurons, are activated and transfer the pain signal to the dorsal horn of the spinal cord. Here the ascending first-order neuron (primary sensory neuron) meets the ascending second order neuron (secondary sensory neuron) and the pain impulse is transmitted through substance P release in the synapse (**Figure 5**, **zoomed in part**). This second-order neuron reaches the sensory cortex via the spinal cord and the brainstem. [17] [27] [28]

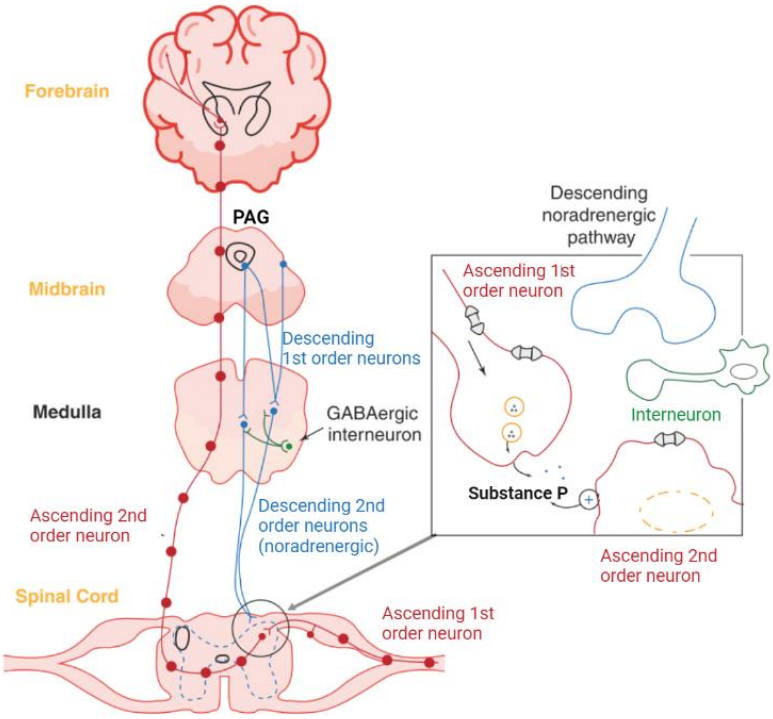

Figure 5: A pain signal activates first-order neurons which transfers the pain signal to a second-order neuron through substance P release in the dorsal horn of the spinal cord. The ascending pain pathway (red line) transfers the pain signal to the sensory cortex. The descending (noradrenergic) pathway (blue line) inhibits the ascending pain pathway (red line) by inhibiting the release of substance P through serotonin or noradrenalin release in the dorsal horn of the spinal cord. GABAergic interneurons inhibit the descending inhibitory noradrenergic pathways in the brainstem in the absence of ascending pain signals. (Adapted from Gupta, Emmanouil, and Sethi, 2020)

The inhibition of pain transmission takes place in the dorsal horn of the spinal cord via the release of various neurotransmitters. To prevent pain perception by the brain, a descending pathway is important (**Figure 5**, **blue neurons**) which can control or inhibit the ascending pathway. This descending pathway originates from the periaqueductal gray matter (PAG) of the midbrain and continues via the pons to the medulla where it transfers a signal to descending second-order neurons in the nucleus raphe magnus. These second-order neurons are serotonergic or noradrenergic and they are important for the inhibition of the ascending pain pathway in the dorsal horn of the spinal cord (**Figure 5**, **zoomed in part**). When these descending second-order neurons release serotonin or noradrenaline in the dorsal horn of the spinal cord, this inhibits substance P release from the first-order neuron of the ascending pathway, preventing pain signals from reaching the brain (**Figure 5**, **zoomed in part**). At the same time interneurons are stimulated to release endogenous opioids or enkephalins, which can also inhibits pain signalling to the brain.[17] [27] [28] The PAG area integrates the ascending pain input with the descending inhibitory output, which is important for the regulation of pain processing in the spinal cord. When there are no ascending pain signals, the descending inhibitory pathways are tonically inhibited by GABAergic interneurons in the brain stem (**Figure 5**, **green neurons in medulla**). This tonic inhibition on these descending inhibitory pathways can be removed through activation of other inhibitory neurons, such as opioid neurons.[27]

Analgesia occurs when someone is unable to feel pain while remaining conscious. When the detection of a painful stimulus is blocked, this is called antinociception. According to the opioid hypothesis, nitrous oxide induces antinociceptive effects by stimulating the release of endogenous opioid peptides from opioid neurons in the PAG area above the spinal cord.[17] Furthermore, nitrous oxide seems to activate noradrenergic neurons in three major

noradrenergic nuclei of the brainstem: the locus coeruleus, the A5 area and A7 area. In order to activate the locus coeruleus, the release of corticotropin-releasing factor (CRF) from the hypothalamus seems to be critical. [29] The following paragraph will elaborate on this.

The inhalation of nitrous oxide causes an increase in the release of endogenous opioids in the PAG. This is hypothesised to occur via the release of CRF from the hypothalamus and/or via the stimulation of nitric oxide (NO) synthesis which provokes the release of endogenous opioids (**Figure 6**, **number 1**).[17] [29] These endogenous opioids bind to opioid receptors on GABA interneurons in both the PAG area and noradrenergic nuclei in the brainstem (e.g. A7) (**Figure 6**, **numbers 2**). GABA interneurons normally tonically inhibit descending noradrenergic neurons, but by the binding of these opioid peptides to opioid receptors on GABA interneurons, the inhibitory GABA interneurons are inhibited themselves (**Figure 6**, **numbers 3**). This means that the excitatory neurons of the descending noradrenergic inhibitory neuronal pathway are no longer inhibited by GABA interneurons and become activated (**Figure 6**, **numbers 4**). The disinhibition of this descending noradrenergic inhibitory neuronal pathway causes a release noradrenalin in the spinal cord, which stimulates two types of adrenergic receptors. These are the α_1 adrenergic receptors located on GABAergic interneurons (**Figure 6**, **number 5**) and α_{2b} adrenergic receptors which are located on the postsynaptic site of the ascending second-order neuron in the dorsal horn of the spinal cord (**Figure 6**, **number 6**). When these two types of receptors are activated, the firing of the ascending second-order neuron is decreased, which results in a reduced ascending pain signalling to the brain (**Figure 6**, **number 7**). So when this ascending second-order neuron is inhibited both directly via noradrenaline and indirectly via GABAergic interneurons, there is a lower pain perception.[29] [30] Both the opioid receptor antagonist naloxone and the GABAa antagonist muscimol inhibit the activity of descending noradrenergic inhibitory neurons, indicating the importance of these two receptors in this mechanism. When these two agents were injected in the three major noradrenergic nuclei of the brainstem (A7, A5 and locus caeruleus) the A7 area seems to be the most important area involved in the antinociceptive effect of nitrous oxide.[27] The role of serotonin in nitrous oxide induced analgesia remains unclear.[17]

In addition to this opioid hypothesis there are other proposed mechanisms for the anaesthetic actions of nitrous oxide. Nitrous oxide is mainly an antagonist of the NMDA glutamate subtype receptor and the main theory on its molecular mechanism underlying anaesthesia is that it noncompetitively inhibits this receptor.

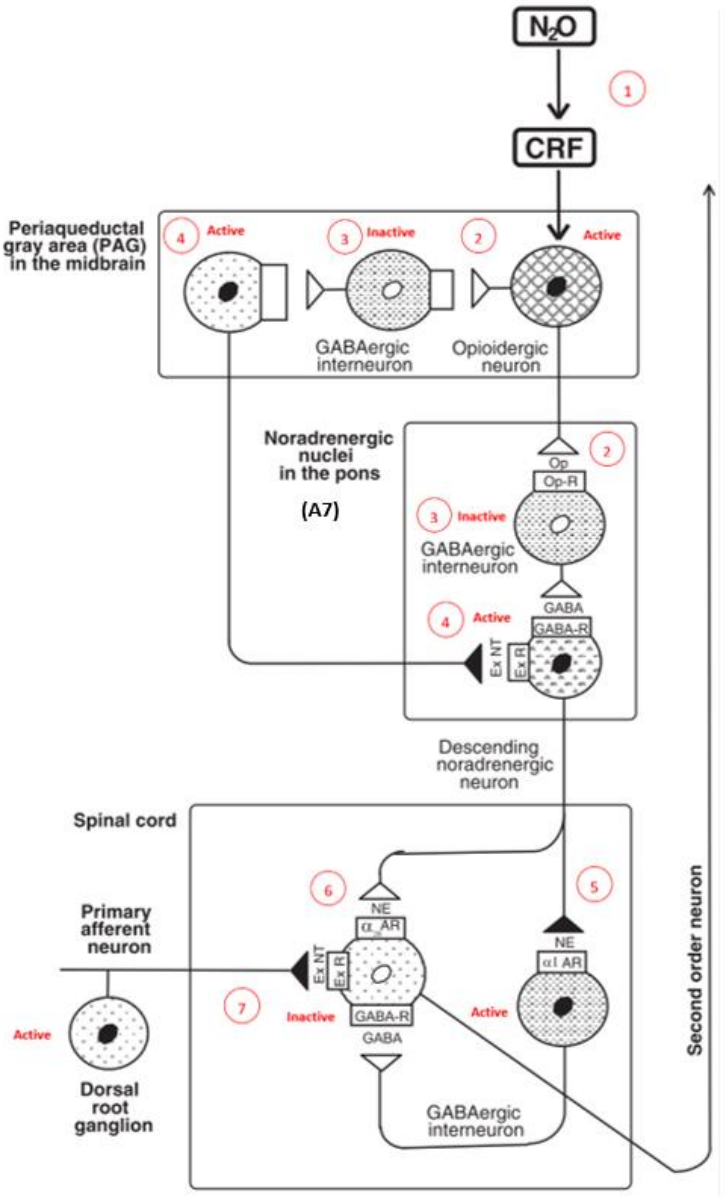

Figure 6: Nitrous oxide (N_2O) activates opioid neurons in the PAG via the release of CRF. Opioid receptors on GABAergic interneurons are stimulated, which causes inhibition of these inhibitory GABAergic interneurons. This causes disinhibition of excitatory noradrenergic descending inhibitory neurons. These noradrenergic neurons release norepinephrine in the spinal cord which stimulates the α_1 adrenergic receptors on GABAergic interneurons or α_{2B} adrenergic receptors situated post-synaptically on the ascending second order neuron. The stimulation of these two adrenergic receptors in the dorsal horn of the spinal cord decreases firing of this second-order neuron and results in a reduction of pain impulses sent to the brain. Black triangle= excitatory synapse; White triangle= inhibitory synapse; NE=norepinephrine; AR=adrenoreceptor; Op= opioid peptides; ExNT= excitatory neurotransmitters; ExR= receptors for excitatory neurotransmitters; (Adapted from Sanders et al., 2008)

The NMDA glutamate receptor in the spinal cord normally has an excitatory role in the CNS and is involved in pain processing. However, when nitrous oxide functions as an NMDA glutamate receptor antagonist, it inhibits excitatory glutamate neurotransmission which is necessary for anaesthesia. The blocking of NMDA receptors by drugs such as nitrous oxide can also protect neurons against NMDA induced neurotoxicity, which happens when NMDA receptors become hyperactivated.[17 26 31] Even though it is speculated that the anaesthetic effect of nitrous oxide results from the blocking of the NMDA receptor, there is still a lot unknown about the molecular and neural pathway involved. For example, it is unclear if non-NMDA glutamate receptors play a role as well.[17 29] It is often suggested that the pharmacology of nitrous oxide resembles that of ketamine, which is also an NMDA receptor antagonist.[17]

Nitrous oxide also has weak effects on the two pore potassium channels as it activates the two-pore-domain potassium channel (TREK-1) channel which is expressed all over the CNS.[15] When this channel is activated, there is an increase in potassium conductance which results in the hyperpolarization of neurons, making it harder for them to fire.[29] Nitrous oxide also seems to inhibit low voltage activated (LVA) or T-type calcium channels, especially the $Ca_v3.2$ channels seem to be important for the analgesic effects. Nitrous oxide is a weak antagonist of serotonin-type 3 receptors and partially inhibits certain nicotinic acetylcholine receptors.[15 30 31] Unlike other anaesthetic agent, however, it seems as though nitrous oxide exerts insignificant effects on GABAa receptors.[29] Furthermore, nitrous oxide has been found to inhibit GABAc receptors.[31]

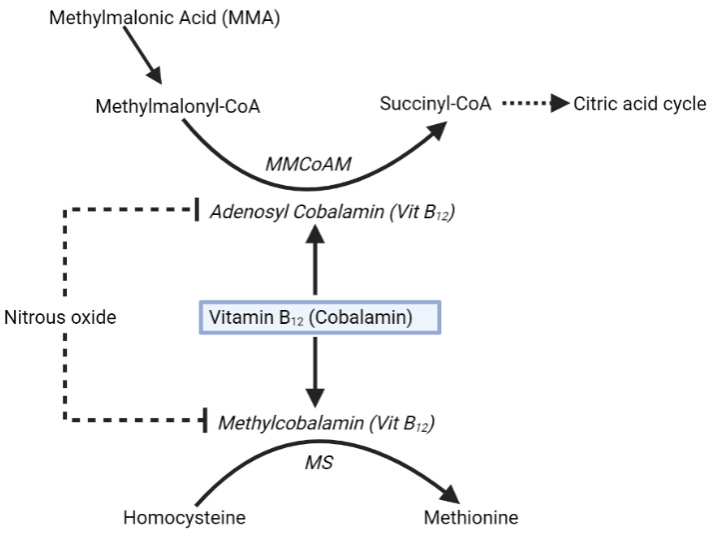

Figure 7: Cobalamin (Vit B_{12}) acts as a coenzyme for methylmalonyl CoA mutase (MMCoAM) and methionine synthase (MS).

The last proposed mechanism involves cobalamin, also known as vitamin B_{12}, which functions as a coenzyme for both methylmalonyl CoA mutase (MMCoAM) and methionine synthase (MS). MMCoAM normally binds to adenosyl-cobalamin and uses it to catalyse the conversion of methyl-malonyl CoA to succinyl CoA which is needed in the citric acid cycle (**Figure 7**).[32] Methionine synthase is an important enzyme for the generation of methyl groups which are essential for the synthesis of DNA, RNA, myelin and catecholamines. Methyl-cobalamin functions as a donor of the methyl group for MS. MS normally binds to methyl-cobalamin and uses it to catalyse the conversion of homocysteine into methionine (**Figure 7**) and for the conversion of 5-methyltetrahydrofolate (5-Methyl-THF) into tetrahydrofolate (THF) in the folate cycle (**Figure 8**). This pathway is important for functioning of cells and a decreased MS activity can cause both genetic and protein abnormalities. [29 32]

However, nitrous oxide oxidizes the B_{12} cobalt ion in cobalamin from a Co^{+} to a Co^{3+} valence state, making it impossible for cobalamin to function as a coenzyme.[3 29] The way the oxidation of cobalamin affects the cobalamin metabolism differs. It can result in the displacement of cobalamin from MS, a decrease in methylated cobalamin, a conversion of cobalamin to its analogue forms, a gradual development of cobalamin deficiency and finally a decrease in MMCoA-mutase activity with a further decrease in MS activity (**Figure 7**).[32]

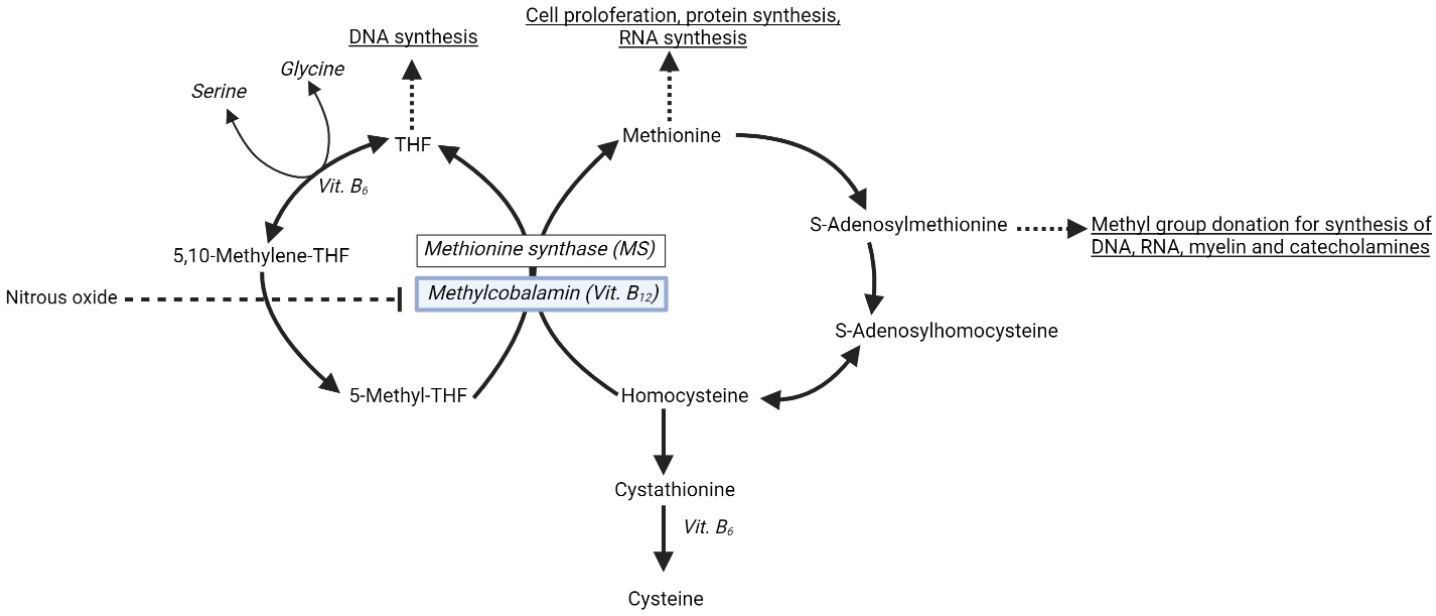

Figure 8: Cobalamin (Vit B_{12}) functions as a coenzyme for methionine synthase (MS) and is crucial for the generation of s-adenosylmethionine and in the folate cycle. Nitrous oxide inhibits cobalamin and in turn inhibits the cycle. 5-methyl-THF = 5-methyltetrahydrofolate; 5,10-methylene-THF = 5,10-methylenetetrahydrofolate; THF = tetra-hydrofolate

The inactivation of cobalamin affects multiple biochemical pathways where cobalamin functions as a cofactor. When there is a cobalamin deficiency, MS cannot convert homocysteine into methionine, which results in increased homocysteine levels and a decrease in myelin production. Furthermore, conversion of 5-methyl-THF into THF is impaired, which impacts DNA synthesis (**Figure 8**). Finally, MMCoAM is unable to convert methyl-malonyl CoA to succinyl CoA, causing an increase in methylmalonic acid (MMA) (**Figure 7**).[3] When nitrous oxide is used as laughing gas, there is a strong increase in MMA levels even though cobalamin levels stay the same (**Background 3**).[23] A shortage in cobalamin can lead to neurological damage, less DNA synthesis, possible megaloblastic anaemia and a disturbed citric acid cycle.[23] The concentration and duration of nitrous oxide exposure are important factors for its effect on cobalamin metabolism.

Background 3: Diagnosis of cobalamin deficiency

Even though the oxidation of the cobalamin cobalt ion makes it inoperable as a coenzyme, a deficiency in cobalamin should not be measured via total and active cobalamin concentrations. It has been shown that these concentrations are not always lower after nitrous oxide usage. Instead levels of homocysteine (HCY) and methylmalonic acid (MMA) are more accurate and reliable measures of a cobalamin deficiency (**Figure 7**).[3] The concentration of these markers increases when there is not enough functional cobalamin. One disadvantage of these markers is that they are expensive and have a long lead time, making it hard to implement it in clinical practice.[23] A way of determining the cause of cobalamin deficiency is by using a Schilling test.[3]

According to the opioid hypothesis, nitrous oxide induces analgesia by stimulating the release of opioid peptides in the PAG via the release of CRF. These opioid peptides indirectly inhibit second-order neurons in the dorsal horn of the spinal cord, causing a lower perception of pain. Nitrous oxide has also been proposed to act as an NMDA glutamate receptor antagonist to induce anaesthesia. Finally, nitrous oxide causes a cobalamin deficiency by oxidizing the B_{12} cobalt ion in cobalamin. This can result in a disturbed myelin production, altered DNA synthesis and it increases the amount of MMA. A shortage in cobalamin could lead to neurological damage but the concentration and duration of nitrous oxide exposure should be kept in mind.

Chapter 4: Effects on the brain

To gain a better understanding of the neurological effects of nitrous oxide, this chapter will highlight some papers focussing on its effects on the brain.

A human study used the EEG topography to quantify the effects of nitrous oxide on brain activity (**Background 4**). Healthy male subjects received either 20% or 40% nitrous oxide for 15 minutes before and during EEG recordings and the EEG also recorded the discontinuation of nitrous oxide. They found that both 20% and 40% nitrous oxide clearly reduced total power, especially at the frontal-vertex sites of the brain, which is the frontal midline of the brain. Furthermore, the inhalation of 20% nitrous oxide was associated with a reduction in delta band power, especially at the frontal-vertex sites. The inhalation of 40% nitrous oxide was associated with an even greater reduction in delta band power at the frontal vertex sites. This causes delta band power to become relatively more dominant at the occipital lobe at the back of the head. This data indicates that the main part of reduction in total power seems to be caused by the suppression of delta band activity. Where general anaesthetics usually promote low-frequency delta band activity, nitrous oxide on the other hand seems to suppress it. It seems as though general anaesthetics disrupt different global brain networks compared to nitrous oxide to induce unconsciousness. When nitrous oxide inhalation was discontinued and nitrous oxide was washed out, there was a selective increase in frontal theta power which even exceeded baseline values.[33]

Another human study used a higher dose of 50-70% nitrous oxide in human subjects and they did not find a reduction in delta band power but instead they found large-amplitude slow-delta oscillations which persisted for 2-12 minutes. Furthermore, the initial effects observed after a high dose of nitrous oxide included a decrease alpha power and a decrease in beta-gamma. Later on, these slow-delta oscillations switched to an increase in beta and gamma oscillations, something which is commonly associated with nitrous oxide use. The writers suggested that this observed increase in slow-delta oscillations might be a result of nitrous oxide antagonizing the NMDA glutamate receptor which would block the most important excitatory input from the brainstem to the thalamus and the cortex.[34] [35] It seems as though the concentration of nitrous oxide plays an important role in its effect on the brain. This is also suggested by another paper which found that lower concentrations of nitrous oxide seemed to attenuate alpha oscillations while at higher concentrations the activity in theta and delta oscillations appears.[36] [37]

Background 4: Electroencephalography (EEG)

Electroencephalography (EEG) is a medical imaging technique which can be used to noninvasively examine the brain and the processes that take place. During an EEG, metal electrodes are placed on the surface of the scalp to record electrical activity. During synaptic excitations of dendrites, neurons in the brain are activated and this produces a local current which can be measured during an EEG. Both normal and abnormal electrical activity can be measured for medical but also for research purposes. An EEG measures oscillating electrical voltages also known as brain waves and these can be quantified by the number of oscillations per second. [37] For humans there are five widely acknowledged brain waves, presented as bands, each with a different frequency and distinct characteristics.[37]

The Gamma (γ) band (> 35 Hz)
→Someone is concentrated or solving problems.
The Beta (β) band (12-35 Hz)
→ Someone is busy, active, relaxed or shows external attention.
The Alpha (α) band (8-12 Hz)
→Someone is very relaxed or shows passive attention.
Theta (θ) band (4-8 Hz)
→Someone is deeply relaxed or has an inward focus.
The Delta (δ) band (0.5-4 Hz)
(Also known as slow-wave activity).
→Someone is asleep or dreaming.

Despite these findings, another paper which used both 30% and 50% of nitrous oxide in combination with EEG in humans subjects, did not find a significant effect on the delta frequency band. They did show that the power in the lower frequency bands increased as a sedative state was induced by nitrous oxide which was reversed after discontinuation of inhalation. Overall the delta frequency band seems to play a role in nitrous oxide induced sedation. However, it is hard to establish the exact effect of nitrous oxide on human brain activity. Research on nitrous oxide induced alterations in human brain activity is scarce and this needs to be investigated more extensively to completely understand these alterations in brain activity.

When looking at changes in the brain on a more cellar level, it has been suggested that nitrous oxide protects the brain from NMDA induced neurodegeneration and it also seems to inhibit NMDA-induced currents in cultured hippocampal neurons.[25] This is in line with an animal study which found that nitrous oxide can protect the brain against excitotoxic neurodegeneration. They administered N-methyl-D, L aspartic acid (NMA, a racemic form of NMDA) to rats which resulted in excitotoxic lesions in the arcuate nucleus (AN) of the hypothalamus. Then they exposed them to a concentration of 117% nitrous oxide for 3 hours under hyperbaric conditions. The size of this lesion in the AN was reduced after rats were treated with nitrous oxide in a dose-dependent manner (**Figure 9; squares, left axis**).[26]

Next to neuroprotection, nitrous oxide has also been reported to increase cell proliferation. An animal study exposed rats to 70% nitrous oxide and assessed cell proliferation in the dentate gyrus of the hippocampus. On the first day the rates of hippocampal cell proliferation were similar between nitrous oxide treated rats and the sham group. However, proliferation had increased by 1.4 folds at day seven after one session of nitrous oxide inhalation. The rate of hippocampal cell proliferation had even increased to two folds after multiple exposures to nitrous oxide. Subanaesthetic doses of nitrous oxide increased cell proliferation in the hippocampus which suggests an increase in neurogenesis.[38]

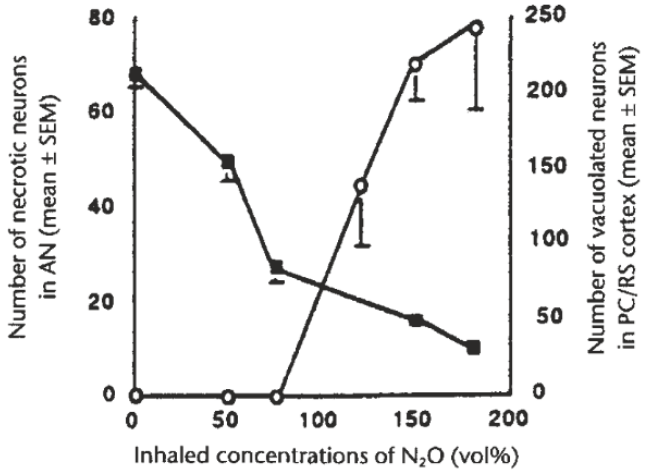

Figure 9: Increasing concentrations of nitrous oxide (N_2O) protect neurons against NMA excitotoxicity by decreasing the size of lesions in the AN in rats presented as mean (±SEM) number of necrotic neurons per section (squares, left y-axis). Nitrous oxide dose-dependently causes vacuolation in PC/RS neurons in rats, presented as mean (± SEM) number of vacuolated neurons per section (circles, right y-axis) with EC_{50} of 117 vol%.

However, higher anaesthetic concentrations of nitrous oxide can also become neurotoxic. The same animal study from before which found that exposing rats to 117% nitrous oxide for 3 hours caused cerebrocortical neurotoxicity in rats. This was observed as vacuole formation in specific pyramidal neurons of the posterior cingulate/retrosplenial cortex (PC/RSC) by swelling of the mitochondria and dilation of endoplasmic reticulum (**Figure 9; circles, right axis**). Nevertheless, this was resolved after 3 hours and the neurotoxicity was reversible.[26]

A follow up study was conducted to further investigate how quickly the neurotoxicity in the PC/RS cortex was reversible after either short or long exposure using a high concentration of nitrous oxide (150%) in rats. After a short term exposure of 3 hours they found that the neurotoxic vacuole reaction in the PC/RS cortex was quickly reversible, meaning that within 3-6 hours the vacuolated neurons had already disappeared. After a long exposure of 16 hours they found that the neurotoxic vacuoles were still visible 24 hours later, which was speculated to be a sign of irreversible neurodegeneration. This was confirmed by an experiment which showed that prolonged exposure (8 hours or more) to nitrous oxide caused neuronal cell death.[39]

This previously mentioned concentration of 117% nitrous oxide in rats compares to 81.9% nitrous oxide in humans when using MAC as an index. As mentioned in Chapter 2, nitrous oxide concentrations cannot exceed 75% under normobaric conditions and this concentration of 81.9% nitrous oxide is similar to the 75%

concentration commonly used for anaesthesia.[26] This concentration of 75% nitrous oxide can only induce partial anaesthesia which calls for the need of additional anaesthetic drugs. One of these drugs is the intravenous anaesthetic ketamine, which is the only other NMDA antagonist next to nitrous oxide, used for modern anaesthesia. A follow up animal research investigated if coadministration of ketamine and nitrous oxide causes an interaction which increases the risk for neurotoxicity. They exposed female rats to increasing concentrations of ketamine (20-80 mg/kg) and nitrous oxide (0-200%), partially under hyperbaric conditions, and they assessed the number of vacuolated neurons in the PC/RS cortex. They showed that the individual administration of either nitrous oxide or ketamine induced dose-dependent neurotoxicity in this brain area by increasing numbers of vacuolated neurons in the PC/RS cortex (**Figure 10A**). When the two substances were combined the neurotoxicity was clearly augmented compared to administration of the individual drugs. It was even the case that ineffective concentrations of either ketamine or nitrous oxide combined, caused severe neurotoxicity in the cerebral cortex of a rat (**Figure 10B**). This research also found that the administration of GABAergic agents like diazepam, halothane and isoflurane are able to protect the brain against this previously mentioned neurotoxicity by NMDA antagonists such as nitrous oxide.[25]

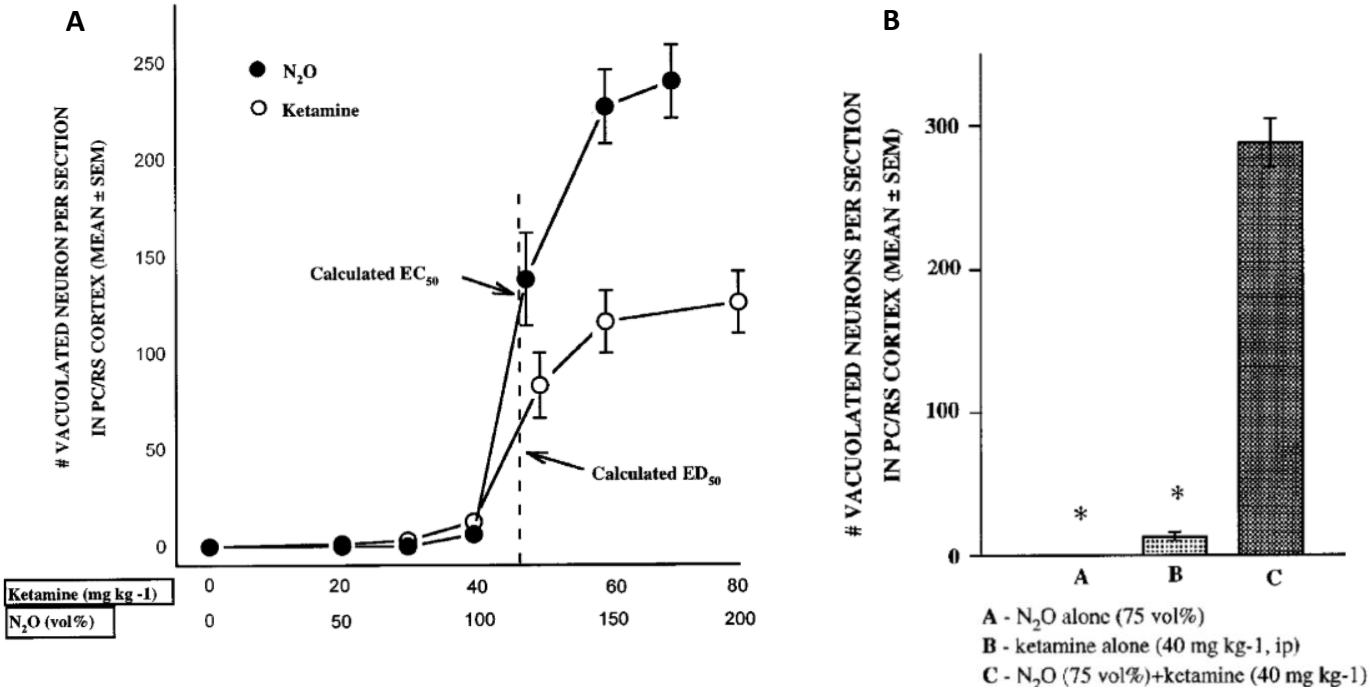

Figure 10: Neurotoxicity (vacuolated neurons) caused by exposure of 3h to nitrous oxide (N_2O; vol%) and ketamine (mg kg^{-1}) in PC/RS cortex neurons of adult rats. A) Separate administration of substances in a dose-dependent manner causes neurotoxicity. B) Separate ineffective concentrations of N_2O (75 vol%) or ketamine (40 mg kg^{-1}) induced no neurotoxicity (A; B) but combined they did (C). EC_{50}: Half maximal active concentration; P<0.0005

Nitrous oxide has been reported to induce psychotic symptoms in already vulnerable individuals.[40] This could be explained by this observed neurotoxicity in the PC/RS cortex loci of the brain since these loci are speculated to be responsible for the ethology of schizophrenia and for othe psychomimetic effects.[41] It remains unknown if this observed neurotoxicity also occurs in the human brain after exposure to both nitrous oxide and ketamine, but if this were the case it would be even more dangerous to use nitrous oxide in combination with ketamine.[25]

Dopaminergic neurons of the ventral tegmental area (VTA) project onto the nucleus accumbens (NAc) in the mesolimbic dopamine system (MLDS). This system is important for the neurobiology of reward for drugs of abuse and it is involved in the aetiology of psychosis. The NMDA receptor antagonist ketamine is associated with activation of VTA dopaminergic neurons and NAc dopamine release. An animal study investigated the effect of nitrous oxide on both spontaneous and ketamine-induced extracellular dopamine levels in the NAc. They exposed rats to 60% nitrous oxide and 40% oxygen for 60 minutes or 70 minutes when ketamine was administered. Nitrous oxide exposure alone resulted in a progressive increase of dopamine levels which gradually declined back to basal levels when nitrous oxide administration was discontinued. Nitrous oxide exposure did

not affect ketamine-induced increased dopamine levels. This shows that nitrous oxide increases NAc dopamine levels but does not interfere with ketamine-induced effects. It remains unknown if this increase in dopamine levels is a result of an increase in dopamine or a decrease in the re-uptake of dopamine. However, since NMDA receptor antagonists activate dopamine neurons in the VTA and nitrous oxide is known to be an antagonist of the NMDA receptor, it is more likely that there is an increase in dopamine release in the NAc rather than a decrease in dopamine re-uptake as a consequence of both ketamine and nitrous oxide.[42] Animal research shows that addictive drugs have the tendency of increasing extracellular dopamine in the NAc.[43] This means that the observed increase in dopamine levels in the NAc after exposure to nitrous oxide could be an indicator that nitrous oxide is a possible addictive agent. This aspect will be discussed further discussed in the next chapter.

The exact effects of nitrous oxide on brain activity remains unclear, although it seems have an influence on slow-delta oscillations in humans. On a more cellular level, nitrous oxide seems to protect the brain against excitotoxic neurodegeneration and low concentrations of nitrous oxide seems to induce cell proliferation in the dentate gyrus of the hippocampus. However, when these concentrations become too high it can cause neurotoxicity in the PC/RS cortex loci. Short term exposure to higher concentrations of nitrous oxide is quickly reversible but prolonged exposure can lead to neuronal cell death in the PC/RS cortex. When nitrous oxide is combined with ketamine, it causes an increase in neurotoxicity in these brain loci, even when the doses of these individual drugs were ineffective. This would make it unadvisable to use nitrous oxide and ketamine at the same time. Nitrous oxide does not seem to enhance ketamine-induced dopamine levels in the NAc, which is an important part of the reward system in the brain. However, nitrous oxide on its own does increases the dopamine levels in the NAc, which might indicate an addictive effect which will be discussed in the next chapter.

Chapter 5: Addictive potential

Addiction treatment centres have not yet reported cases of (pure) nitrous oxide addiction which would suggests that the gas could be seen as a non-addictive substance.[21] However, an animal study in the previous chapter reported that exposure to nitrous oxide increased dopamine levels in the nucleus accumbens, a phenomenon which is normally associated with addictive substances. This chapter will therefore look into the addictive potential of nitrous oxide.

The conditioned place preference (CPP) paradigm is an animal model used to measure reward-related behaviours. This paradigm measures the associations between a (possible) rewarding stimulus and a certain environment. This paradigm is based on the idea that a preference (or aversion) is obtained for an environmental context following classical conditioning where the experience of an animal with a certain drug is paired with a context. Basically, if a drug has rewarding effects then the animal prefers the drug-associated environment and if the drug has an adverse effect then the animal avoids this environment.[24] The paradigm uses an apparatus consisting of two or three compartments, with different contextual characteristics like a pattern or texture. During the first phase of the CPP paradigm, the animals habituate to the compartments, to get used to the environment and to measure the baseline preference of the animals for the compartments. The second phase of the paradigm consists of conditioning sessions. Before or during this second phase, the animal receives the drug or vehicle (control) via injection or inhalation. The animals are then placed and kept in a distinct contextual compartment to acquire drug-context associations. During the final phase of the paradigm the CPP test takes place during which the animals are given free access to all compartments and the amount of time spent in the drug-paired compartment is measured.[44]

An animal study measured the rewarding or aversive effects of nitrous oxide using this CPP model. The experimental group consisted of 103 male Long-Evans rats which were exposed to different concentrations of nitrous oxide (8%, 15%, 30% or 60%) in one chamber and to a placebo gas in another chamber with both chambers containing different contextual characteristics (clear and striped). The control group received placebo gas in both chambers. The gas exposure took place 40 minutes per day for 8 consecutive days (conditioning phase). On day 9 there was a 20 minute test session when placebo gas was delivered to both chambers and the animals could move freely between chambers to measure the preference of the animal for the drug (**Figure 11A**). This was followed by a 20 minute test session on day 10 when nitrous oxide was delivered to both chambers to check for a state-dependent memory effect (**Figure 11B**). In one group the increasing concentrations of nitrous oxide were paired to the clear chamber (*circles*) and in another group nitrous oxide was paired to the striped chamber (*squares*) during the conditioning phase. The time spent in the clear chamber was measured for both groups and for all 4 concentrations of nitrous oxide. The more time spent in the chamber paired with nitrous oxide, the higher the preference for nitrous oxide and the less time spent in this chamber, the higher the aversion for nitrous oxide. They found a conditioned place aversion for the chambers that had been paired with 30% or 60% nitrous oxide on both day 9 (Placebo test; **Figure 11A**) and day 10 (N_2O test; **Figure 11B**). A conditioned place preference was found for the environment paired to 8% nitrous oxide especially on day 10 (**Figure 11B**).[24]

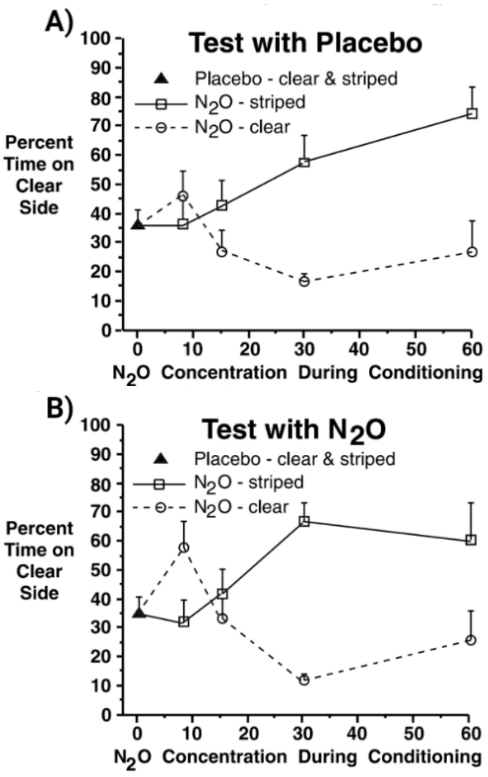

Figure 11: Mean (±SEM) percentage of time spent on clear side. Rats exhibited a conditioned place aversion to the environment previously paired with 30% and 60% nitrous oxide (N_2O) during both A) Placebo test sessions (day 9) and B) N_2O test sessions (day 10). A conditioned place preference was observed especially during the N_2O test session for the environment previously paired with 8% N_2O. Triangles= placebo group; Squares= N_2O paired to striped chamber; Circles= N_2O paired to clear chamber. (Adapted from Ramsay et al., 2003)

Another animal model to study addiction uses the concept of self-administration procedures. During these procedures an animal performs an action, like pressing a lever, in order to deliver a dose of a drug via an intravenous catheter or via inhalation. The most important assumption of this procedure is that drugs work as reinforcers, meaning that they are likely to increase the behaviour that produces them.[45]

An animal research looked at nitrous oxide self-administration using four male rats. They used a special designed apparatus of four chambers where rats could move in between chambers containing different concentrations of nitrous oxide (30% and 60%) or placebo gas. The self-administration behaviour was measured as the amount of time a rat spent in a specific concentration of nitrous oxide. The concentrations of nitrous oxide alternated in between chambers. The first experiment consisted of a "forced-choice" procedure where rats could only move between these four chambers. When a rat showed self-administration behaviour, it would spend more time in the nitrous oxide chamber. When a rat wanted to avoid nitrous oxide it would go to the placebo chamber. In this experiment two rats selected the nitrous oxide chambers, one rat seemed to avoid nitrous oxide and the last rat did not move between chambers showing no preference or avoidance for nitrous oxide. The reversal design of alternating gas concentrations between gas chambers made it sometimes difficult to interpret self-administrating behaviour when a rat stayed in the same room. This was the reason why they conducted a second experiment which consisted of a "free-choice" procedure were the rats started in a central chamber of the apparatus from which the animal could enter the separate gas chambers. With this design animals are not being forced to choose between nitrous oxide or placebo, but they can also choose for a no drug environment with room air: the central chamber. During this experiment the same two rats as before showed a clear preference for the nitrous oxide chamber, the same rat avoided the nitrous oxide chamber and the same rat had no preference or aversion to nitrous oxide by spending most of the time in the central chamber.[24] This experiment seems to show some conflicting results as half of the rats (N=2) showed self-administration behaviour in both the "forced-choice" and the "free-choice" procedure while this self-administration behaviour was not observed in the other two animals.

Reinforcement and rewarding effects can also be assessed via intracranial self-stimulation (ICSS) in mice. For this procedure, electrodes are implanted in the medial forebrain bundle of mice, causing a pleasure sensation as a consequence of electrical pulses. When the mice then performs an operant response (e.g. pressing a lever) they receive these electrical pulses. This study trained these mice using a rate-frequency ICSS procedure during which the frequency of the stimulation varied whereas other parameters like duration and intensity were kept constant. Most drugs with the potential of abuse facilitate behaviours reinforced by ICSS. In this case the behaviour was the pressing of a lever which resulted in electrical stimulation. They tested the effects of different nitrous oxide concentrations (20%, 40%, 60% and 80%) on ICSS. The concentration of 40% nitrous oxide facilitated ICSS, but only two out of ten intermediate stimulation frequencies showed a significantly greater percentage of maximum control response (% MCR; **Figure 12A).** On the other hand, concentrations of 60% and 80% nitrous oxide suppressed ICSS as the % MCR was lower compared to the control group for one (**Figure 12B**) and five (**Figure 12C**) frequencies respectively. This suggests that nitrous oxide only weakly facilitates ICSS responding in a rate-frequency procedure compared to controls. Higher concentrations of nitrous oxide seem to suppress ICSS. As drugs with the potential for abuse, facilitate behaviour reinforced by ICSS, it could be speculated that nitrous oxide's potential for abuse is thus low.[46]

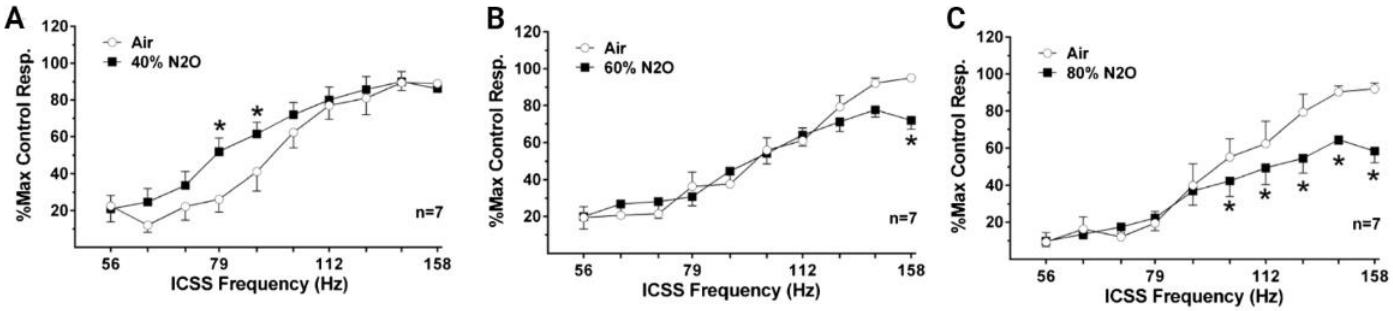

*Figure 12: Mean (±SEM) percentage of maximal control ICSS response rate following 20 minute of exposure to nitrous oxide gas combined with 100 % oxygen. A) 40% nitrous oxide slightly facilitates ICSS compared to control. B) 60% nitrous oxide supresses ICSS compared to control. C) 80% nitrous oxide suppresses ICSS compared to control. Closed squares= nitrous oxide; Open circles= Air (control) *p<0.05*

Self-administration with nitrous oxide was also tested in primates. Four squirrel monkeys were taught to associate the pressing of a key to the administration of nitrous oxide via a helmet placed on their head. The pressing of the key could not be maintained at comparable levels when nitrous oxide was absent. This indicated that nitrous oxide can function as a reinforcer. With fixed-ratio schedule, when more than a single response is needed before nitrous oxide is delivered, the key pressing behaviour was maintained in strength. Response rates increased with an increasing fixed-ratio, which shows similarities between nitrous oxide delivery and delivery of other reinforcers. When a single response was needed for reinforcement, the concentration-effect curves indicated that a beyond a certain concentration, which was necessary to maintain the behaviour, little effect was seen on the performance. Overall this study seems to indicate that nitrous oxide could be seen as a reinforcer.[47]

There is even a human research where human subjects get the option to self-administer 30% nitrous oxide, 100% oxygen or drug-free air for 10 minutes without knowing which is which. They showed that the choice of the subjects differed between subjects but remained constant within an individual. Five out of twelve subjects consistently chose nitrous oxide, four subjects consistently avoided nitrous oxide and three subjects were undecisive between nitrous oxide and oxygen/placebo. This research emphasises the need for measuring multiple characteristics when assessing the abuse potential of this drug.[48]

Next to reward and reinforcement, a drug with an addictive potential can be recognised by its withdrawal symptoms. When a drug has managed to induce dependence after chronic use, a withdrawal reaction is visible when the drug ingestion is stopped abruptly. In mice this is observed when a mouse is picked up by its tail as convulsions (seizures). An animal experiment showed that these withdrawal seizures were visible after short exposure to anaesthetic levels of nitrous oxide or after chronic exposure to subanaesthetic levels of nitrous oxide. A follow up experiment showed that in mice a minimum of 0.5 atmospheric pressure (atm) was needed to show convulsion and these convulsions were visible in almost all mice after exposure of 0.9 atm. They showed that the partial pressure of nitrous oxide seems to have a far greater impact on the incidence of convulsions than the duration of exposure (**Figure 13**). The duration of exposure after 15 to 30 minutes did not increase the incidence of convulsions regardless of the concentration of nitrous oxide (data not shown). The dependent stage was however very reversible as the withdrawal reaction did not persist for longer than 90 minutes (**Figure 13**).[49] These withdrawal symptoms provide evidence of physical dependence after nitrous oxide exposure in mice. The duration of these symptoms is however limited and partial pressure seems to be important for the incidence of convulsions.

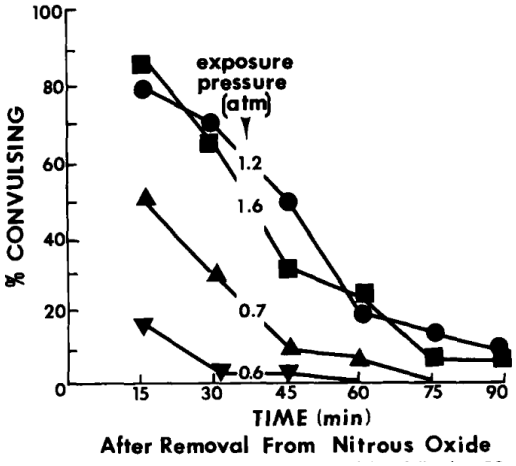

*Figure 13: Average percentage of convulsing in mice following varying times of exposure to nitrous oxide. Each line shows a different partial pressure of nitrous oxide. Higher partial pressure increased the duration of withdrawal reaction as shown by % convulsing. Square= 1.6 atm; circle= 1.2 atm; triangle upwards= 0.7 atm; triangle downwards= 0.6 atm. *p<0.05*

In contrast to inducing withdrawal symptoms, nitrous oxide has also been reported to reduce withdrawal from other abusive substances like alcohol, nicotine, cannabis and opiates. It has even been reported to reduce craving during acute withdrawal, making it a possible treatment for addiction. An animal study tested the ability of nitrous oxide to suppress alcohol consumption. They used rats which were genetically selected for either High Alcohol Drinking (HAD) or Alcohol-Preferring (P). The exposure to nitrous oxide gas, which contained 75% nitrous oxide and 25% oxygen, lasted for either 30 minutes, 60 minutes or 120 minutes. The control group received pure oxygen for 120 minutes. They showed that nitrous oxide suppressed the consumption of ethanol one hour after gas exposure, but not 25 hours after gas exposure in both HAD and P rats (**Figure 14**). The P rats showed significantly lower alcohol consumption one hour after gas exposure for all three individual durations compared to 23 hrs before exposure. The HAD rats showed significantly lower alcohol consumption one hour after gas exposure for 60 and 120 minutes of exposure compared to 23 hrs before exposure. They also showed that nitrous oxide did not suppress water drinking, indicating that nitrous oxide has a specific effect on ethanol consumption. Nitrous oxide could be useful as an acute treatment for alcohol craving during withdrawal.[50]

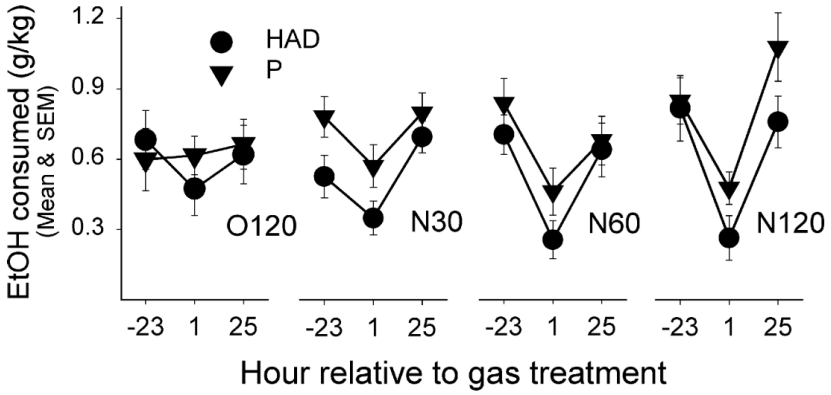

Figure 14: Ethanol (EtOH) consumption (g/kg) as mean (±SEM) by High Alcohol Drinking (HAD; circles) and Alcohol-Preferring (P; triangles) rats 23 h before, 1 h and 25 h after nitrous oxide treatment. Rats were exposed to pure oxygen for 120 min (O120) or to nitrous oxide for 30 min (N30), 60 min (N60) or 120 min (N120). EtOH consumption was suppressed 1h after treatment after N30 (P animals only) and after N60 and N120 (both HAD and P animals). EtOH consumption was not suppressed 25 h after any nitrous oxide treatment. (Adapted from Kosobud et al., 2009)

The previous mentioned CPP paradigm was also used in an experiment to measure the effect of nitrous oxide as a treatment for drug abuse. They investigated the effect of nitrous oxide on the acquisition and expression of conditioned place preference induced by morphine and cocaine. When measuring acquisition of CPP, the drug should be administered during the conditioning phase where the drug is paired to the context. When measuring expression of CPP, the drug should be administered during the CPP test.[44] Mice were exposed to either morphine or cocaine in one compartment and to saline in the other compartment. This was followed by exposure to 50% nitrous oxide exposure either during the conditioning phase or during the CPP test. The results showed that nitrous oxide itself did not induce CPP in these mice but it did impair the *acquisition* of morphine-induced CPP. Nitrous oxide also blocked the *expression* of morphine and cocaine induced CPP. Nitrous oxide itself did not induce changes in behaviour like anxiety, depression of locomotion.[51] This suggest that nitrous oxide could have a clinical beneficial effect on morphine and cocaine addiction management.

Even if it has abusive potential, it remains difficult to determine the degree of this potential. Is it more dangerous than an alcohol addiction or would it be an option to restore an alcohol addiction with nitrous oxide usage if this is less dangerous? The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is a handbook which contains descriptions, symptoms and criteria for the diagnosis of mental disorders. The DSM-5 does not categorize nitrous oxide disorders among Substance Use Disorder (SUD) as a consequence of inhalant agents (SUD-I). Instead nitrous oxide is classified under “Other” (unknown) substance-related disorders in the DSM-5. This last category contains drugs of which the degree and range of the induced disorders are uncertain. This means that there is not enough know about the degree of nitrous oxide abuse to classify it among SUD-I agents in the DSM-5.[52]

The conflicting results regarding the rewarding system of nitrous oxide is visible in all these researches. Rats in the first CPP study showed aversion to higher concentrations and preference for a lower concentration of nitrous oxide. The next study using four rats showed inconsistent results of self-administration between animals, while the animals stayed consistent within the experiment. This phenomenon was also observed in a human experiment where the choice for different gasses varied across subjects (between-subject variability) but remained constant within a subject. The experiment with squirrel monkeys seems to indicate that nitrous oxide works as a reinforcer but this seems to be contradicted by the ICSS experiment. The withdrawal experiment seems to support the abusive potential of nitrous oxide showing that already a short exposure of a minimum of 0.5 atm is enough to show convulsion. So it is not easy to conclude that nitrous oxide has the potential be an addictive drug. However, there seems to be evidence that nitrous oxide has the potential to function as a treatment for alcohol and morphine/cocaine addiction and withdrawal.

Discussion

Nowadays, nitrous oxide is often used as an anaesthetic agent for surgery often in combination with other treatments or as an analgesic and anxiolytic agent by dentists. For these purposes, nitrous oxide is considered to be a safe gas. However, the gas has gained popularity over the last couple of years especially among teens and students as a recreational drug due to its "high" feeling and quick onset. The consequent increase in reports of neurological complaints and disturbances in sensory perception such as tingling of the hands and legs, is a reason for concern.

The low blood/gas partition coefficient of nitrous oxide causes the gas to be rapidly taken up in the blood and quickly transported to the brain causing a quick onset of effects. However, this low solubility of nitrous oxide also results in a faster recovery from its effects. Therefore, inhaling small amounts of nitrous oxide seems relatively safe at first, as the effect wears of rather quickly at lower concentrations.[15] Once nitrous oxide enters the body, it has been hypothesized to exert various effects. According to the opioid hypothesis, nitrous oxide induces antinociceptive effects through stimulating the release of opioid peptides in the PAG. Furthermore, the gas has been hypothesized to antagonize the NMDA glutamate receptor necessary to induce anaesthesia and protecting neurons against NMDA induced neurotoxicity. It is important to keep in mind that these are still speculations as the exact molecular and neural pathway involved remain uncertain. Finally, it has been found that nitrous oxide oxidizes the B_{12} ion in cobalamin (vitamin B_{12}), making it inactive as a coenzyme for MMCoA and MS resulting in neurological damage, less DNA synthesis, megaloblastic anaemia and a disturbed citric acid cycle.

The exact effects on the gas on the brain remain unclear, however, the delta frequency band seems to play a role in sedation. On a more molecular level, the gas seems to prevent NMDA induced neurodegeneration both in cell cultures and animal models. Next to this neuroprotection, low concentrations of nitrous oxide have been suggested to increase cell proliferation, at least in the dentate gyrus of the hippocampus. When the concentration of nitrous oxide increases, it can become neurotoxic, as was observed as vacuole formation in the PC/RS cortex of a rat brain. This effect was quickly reversible when the exposure to this high concentration lasted for a short period, meaning that the vacuoles disappeared rather quickly. Longer exposure on the other hand caused irreversible neuronal cell death. When the gas is administered together with ketamine, it increased neurotoxicity in the PC/RS cortex. Finally, nitrous oxide exposure increased NAc dopamine levels without interfering with ketamine-induced effects most likely via an increase in dopamine release.

To see if this increase in dopamine indicates an addictive potential, nitrous oxide administration was tested in various behavioural animal experiments. A CPP study with rats indicated that higher concentrations seemed to cause a preference and lower concentrations an aversion for nitrous oxide. Another study using self-administration showed inconsistent results in rats which was also observed in humans. In a similar experiments using primates, the gas seemed to function as a reinforcer. When looking at withdrawal symptoms, nitrous oxide seems to have abusive potential by inducing convulsion. But despite these negative effects of nitrous oxide, it is also shown to have a potential in treating alcohol and morphine/cocaine addiction and withdrawal. In literature, nitrous oxide has also been suggested as a treatment for certain psychiatric conditions and to function as an antidepressant.[53] Overall it is difficult to conclude if nitrous oxide has an abusive potential as some conflicting results indicate an aversion for nitrous oxide instead of a preference. However, the concentration of nitrous oxide seems to influence the outcome of some experiments. When comparing nitrous oxide to other non-competitive NMDA receptor antagonists like ketamine, it is considered to have a low addictive potential. The rapid on- and off-set of nitrous oxide gives users the opportunity to resume everyday activities rather quickly removing an important logistical barrier which is observed with ketamine.[15]

On one hand, nitrous oxide does not seem that dangerous as its effects are quickly reversible and it even seems to increase cell proliferation in the hippocampus, prevents NMDA induced neurodegeneration and could function as a treatment for certain addictions and withdrawal symptoms. On the other hand, nitrous oxide induces irreversible neurotoxicity, depletes vitamin B_{12}, functions as a reinforcer, induces withdrawal symptoms and increases ketamine-induced neurotoxicity when exposure was longer or concentrations were higher.

As can be seen in this essay, assessing the biological effects of nitrous oxide for recreational use, depends on multiple factors such as the duration of exposure and the concentration of the drug. Chapters 4 and 5 show how

the duration of nitrous oxide exposure determines how detrimental its effects are and how quickly a person can recover from these effects. When used for recreational purposed, the duration of exposure to the drug also varies between individuals. An example of a case study with a long exposure to nitrous oxide is of a man (21 y.o.) who used 25 bulbs per day for a period of 12 months. He experienced ascending numbness and tingling of the skin in his lower limbs and fingertips. He showed neurodegeneration in the spine from nitrous oxide induced B_{12} deficiency. However, after he was treated with various drugs to restore this deficiency (hydroxocobalamin and methionine) he completely recovered within 6 months.

Another factor is the concentration of nitrous oxide which varies too between individual recreational users. An example of a case study with a high amount of nitrous oxide is of a woman (43 y.o.) who used between 100-200 bulbs per day for 8 consecutive days. She already had a history of using nitrous oxide in a social setting for 12 months prior. She experienced tightness in the chest, dizziness, ataxia and tingling of the skin. Her blood values showed B_{12} deficiency but after administration of hydroxocobalamin she was discharged.[5] A bulb itself contains 10ml of nitrous oxide.[3 21] However, it is unclear what the exact volume concentration of the *inhaled* nitrous oxide out of a bulb is. This depends on factors such as the space in the lungs, mouth breathing, the ventilatory condition of the user and the breathing between inhalation which all cause fluctuations. The exact concentration that is delivered to the alveoli is thus unclear and the maximum concentration used in literature does not exceed 75% under normal pressure at sea level.[25]

It is important to note that these two previously mentioned case studies are extreme cases.[5] In most cases nitrous oxide is used very modestly with more than 90% of consumers using it monthly or less without many reports of neurological effects. This makes it seem relatively safe. The number of bulbs inhaled in a session is usually fewer than 5 and only a small group of heavy users inhales 75-125 bulbs per session to remain under influence.[21] The cases with neurological and haematological toxicities only occur after heavy use or prolonged exposure to a high dose which results in vitamin B_{12} deficiency. Death by nitrous oxide has been reported in a few cases where nitrous oxide was found in the bloodstream. However, these individuals inhaled pure nitrous oxide directly from a tank and died because of suffocation due to hypoxia.[3] Individuals who have generally lower vitamin B_{12} levels have a higher risk for developing vitamin B_{12} deficiency.[3] There is also a lot unknown about the consequences of repeated use of laughing gas at a young age.[7] Because not much is known, minors should be prevented from using nitrous oxide until more is known about its effects on the developing brain. It is thus important to know when you are at risk and what dose and duration are safe when using it.

Because this drug is relatively uncommon to society, it is interesting to look into the regulations of this drug. Regulations seem needed as the recreational use of the gas causes public disturbances (littering, traffic accidents) as well as neurological damage. However, one could wonder what if cigarettes and alcohol were discovered in the present-day. Would these drugs have been accepted by society nowadays with its known detrimental effects on the human body? Or are these drugs only accepted because of its history in society? It can be speculated that alcohol and tobacco are much more harmful to our health compared to laughing gas but because we are so accustomed to these two substances as a society, we allow them to be sold and consumed under certain conditions. Of course there are regulations on age limit and its usage in traffic, but both sustances legal to consume and sell. Society is informed of their risks on the developing brain, during pregnancy and when participating in traffic through education and government funded campaigns. Maybe a solution could be to inform users of nitrous oxide about its potential risks and side effects just like with alcohol and tobacco.

Furthermore it is important to inform users of its risks when combining it with other drugs such as ketamine. Doctors on the other hand should be informed about the treatment of the observed complications as the supplementation of cobalamin can resolve neurological complications relatively quickly. It could be an idea to copy the regulations of alcohol and tobacco, by making the use of the gas legal after reaching a certain age and by making it illegal to use while participating in traffic. When these steps are taken into consideration, maybe it is not necessary to ban the drug by taking (further) legal measures. When laughing gas becomes illegal, it is harder to control the distribution and consumption as it will go onto the black market. This means that the government loses grip and control of its distribution and consumption.

Conclusion

The recreational use of nitrous oxide should not be prohibited based on its biological effects. Instead regulations should be implemented preventing minors from using it and preventing it from being used while participating in traffic. Furthermore, users should be informed about the effects and risks of nitrous oxide just as is being done with users of alcohol and tobacco. This way accidents and abuse can be prevented without losing control over the use and distribution of the substance.

Bibliography

1. Meerdere auto's botsen op A16 bij Breda: snelweg bezaaid met flessen lachgas | 112 & misdaad | bndestem.nl. Accessed October 6, 2021. https://www.bndestem.nl/breda/meerdere-auto-s-botsen-op-a16-bij-breda-snelweg-bezaaid-met-flessen-lachgas~a2c25eba/

2. Al bijna duizend verkeersongelukken in 2019 door gebruik lachgas | NU - Het laatste nieuws het eerst op NU.nl. Accessed October 6, 2021. https://www.nu.nl/binnenland/5977299/al-bijna-duizend-verkeersongelukken-in-2019-door-gebruik-lachgas.html

3. Garakani A, Jaffe RJ, Savla D, et al. Neurologic, psychiatric, and other medical manifestations of nitrous oxide abuse: A systematic review of the case literature. *Am J Addict*. 2016;25(5):358-369. doi:10.1111/ajad.12372

4. Nationale Drug Monitor Jaarbericht 2020 | Rapport | Rijksoverheid.nl. Accessed October 6, 2021. https://www.rijksoverheid.nl/documenten/rapporten/2021/03/09/nationale-drug-monitor-jaarbericht-2020

5. Lightfoot E, Brownlie D, Lightfoot J. Nitrous oxide toxicity: When laughing gas is no laughing matter – A discussion of two cases. *EMA - Emerg Med Australas*. 2020;32(4):710-711. doi:10.1111/1742-6723.13538

6. Hathout L, El-Saden S. Nitrous oxide-induced B12 deficiency myelopathy: Perspectives on the clinical biochemistry of vitamin B12. *J Neurol Sci*. 2011;301(1-2):1-8. doi:10.1016/j.jns.2010.10.033

7. Rombouts M, Van Dorsselaer S, Scheffers-van Schaijck T, Tuithof M, Kleinjan M, Monshouwer K. Jeugd en riskant gedrag 2019. *Kerngegevens uit het peilstationsonderzoek Sch*. Published online 2020:37-53.

8. CAM. Risicobeoordeling lachgas. 2019;(november):66. https://www.rivm.nl/sites/default/files/2019-12/risicobeoordelingsrapport lachgas 20191209 beveiligd.pdf

9. Basisnotitie Coördinatiepunt Assessment en Monitoring nieuwe drugs | RIVM. Accessed October 6, 2021. https://www.rivm.nl/publicaties/basisnotitie-coordinatiepunt-assessment-en-monitoring-nieuwe-drugs

10. Kabinet komt met verbod op recreatief gebruik van lachgas | NU - Het laatste nieuws het eerst op NU.nl. Accessed October 6, 2021. https://www.nu.nl/binnenland/6016477/kabinet-komt-met-verbod-op-recreatief-gebruik-van-lachgas.html

11. wetten.nl - Regeling - Opiumwet - BWBR0001941. Accessed October 6, 2021. https://wetten.overheid.nl/BWBR0001941/2021-07-01

12. Buslov A, Carroll M, Desai MS. Frozen in time: A history of the synthesis of nitrous oxide and how the process remained unchanged for over 2 centuries. *Anesth Analg*. 2018;127(1):65-70. doi:10.1213/ANE.0000000000003423

13. Emmanouil DE, Quock RM. Advances in understanding the actions of nitrous oxide. *Anesth Prog*. 2007;54(1):9-18. doi:10.2344/0003-3006(2007)54[9:AIUTAO]2.0.CO;2

14. Randhawa G, Bodenham A. The increasing recreational use of nitrous oxide: History revisited. *Br J Anaesth*. 2016;116(3):321-324. doi:10.1093/bja/aev297

15. Kalmoe MC, Janski AM, Zorumski CF, Nagele P, Palanca BJ, Conway CR. Ketamine and nitrous oxide: The evolution of NMDA receptor antagonists as antidepressant agents. *J Neurol Sci*. 2020;412(March):116778. doi:10.1016/j.jns.2020.116778

16. Weimann J. Toxicity of nitrous oxide. *Best Pract Res Clin Anaesthesiol*. 2003;17(1):47-61. doi:10.1053/bean.2002.0264

17. Gupta K, Emmanouil D, Sethi A. *Nitrous Oxide in Pediatric Dentistry*.; 2020. doi:10.1007/978-3-030-29618-6

18. Becker DE, Rosenberg M. Nitrous oxide and the inhalation anesthetics. *Anesth Prog*. 2008;55(4):124-131. doi:10.2344/0003-3006-55.4.124

19. Faddy SC, Garlick SR. A systematic review of the safety of analgesia with 50% nitrous oxide: Can lay responders use analgesic gases in the prehospital setting? *Emerg Med J*. 2005;22(12):901-906. doi:10.1136/emj.2004.020891

20. Geneva: World Health Organization. World Health Organization Model List of Essential Medicines. *Ment Holist Heal Some Int Perspect*. 2019;21:23-24.

21. van Amsterdam J, Nabben T, van den Brink W. Recreational nitrous oxide use: Prevalence and risks. *Regul Toxicol Pharmacol*. 2015;73(3):790-796. doi:10.1016/j.yrtph.2015.10.017

22. Tuithof M, Dorsselaer S, Monshouwer K. Middelengebruik onder studenten van 16-18 jaar op het MBO en HBO 2017. 2018;2015.

23. Hendriks E, Jong HK de. Neurologische klachten en diagnostiek bij lachgasgebruik. *Huisarts Wet*. 2020;63(11):57-59. doi:10.1007/s12445-020-0876-2

24. Ramsay DS, Watson CH, Leroux BG, Prall CW, Kaiyala KJ. Conditioned place aversion and self-administration of nitrous oxide in rats. *Pharmacol Biochem Behav*. 2003;74(3):623-633. doi:10.1016/S0091-3057(02)01048-1

25. Jevtovic-Todorovic V, Benshoff N, Olney JW. Ketamine potentiates cerebrocortical damage induced by the common anaesthetic agent nitrous oxide in adult rats. *Br J Pharmacol*. 2000;130(7):1692-1698. doi:10.1038/sj.bjp.0703479

26. Jevtović-Todorović V, Todorović SM, Mennerick S, et al. Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. *Nat Med*. 1998;4(4):460-463. doi:10.1038/nm0498-460

27. Ohashi Y, Guo T, Orii R, Maze M, Fujinaga M. Brain stem opioidergic and GABAergic neurons mediate the antinociceptive effect of nitrous oxide in fischer rats. *Anesthesiology*. 2003;99(4):947-954. doi:10.1097/00000542-200310000-00030

28. Silverthorn DU. Sensory Physiology. In: *Human Physiology, An Integrated Approach*. ; 2016:344-348.

29. Sanders RD, Weimann J, Maze M, Warner DS, Warner MA. Biologic Effects of Nitrous Oxide. *Anesthesiology*. 2008;109(4):707-722. doi:10.1097/aln.0b013e3181870a17

30. Nagele P, Zorumski CF, Conway C. Exploring Nitrous Oxide as Treatment of Mood Disorders: Basic Concepts. *J Clin Psychopharmacol*. 2018;38(2):144-148. doi:10.1097/JCP.0000000000000837

31. Yamakura T, Harris RA. Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels: Comparison with isoflurane and ethanol. *Anesthesiology*. 2000;93(4):1095-1101. doi:10.1097/00000542-200010000-00034

32. Kondo H, Osborne ML, Kolhouse JF, et al. Nitrous oxide has multiple deleterious effects on cobalamin metabolism and causes decreases in activities of both mammalian cobalamin-dependent enzymes in rats. *J Clin Invest*. 1981;67(5):1270-1283. doi:10.1172/JCI110155

33. Foster BL, Liley DTJ. Effects of nitrous oxide sedation on resting electroencephalogram topography. *Clin Neurophysiol*. 2013;124(2):417-423. doi:10.1016/j.clinph.2012.08.007

34. Pavone KJ, Akeju O, Sampson AL, Ling K, Purdon PL, Brown EN. Nitrous oxide-induced slow and delta oscillations. *Clin Neurophysiol*. 2016;127(1):556-564. doi:10.1016/j.clinph.2015.06.001

35. Ryu JH, Kim PJ, Kim HG, Koo YS, Shin TJ. Investigating the effects of nitrous oxide sedation on frontal-parietal interactions. *Neurosci Lett*. 2017;651:9-15. doi:10.1016/J.NEULET.2017.04.036

36. Kawaguchi T, Mashimo T, Yagi M, Takeyama E, Yoshiya I. Xenon is another laughing gas. *Can J Anaesth 1996 436*. 1996;43(6):641-642. doi:10.1007/BF03011783

37. Malver LP, Brokjær A, Staahl C, Graversen C, Andresen T, Drewes AM. Electroencephalography and

analgesics. *Br J Clin Pharmacol*. 2014;77(1):72-95. doi:10.1111/BCP.12137

38. Chamaa F, Bahmad HF, Makkawi AK, et al. Nitrous oxide induces prominent cell proliferation in adult rat hippocampal dentate gyrus. *Front Cell Neurosci*. 2018;12(May):1-8. doi:10.3389/fncel.2018.00135

39. Jevtovic-Todorovic V, Beals J, Benshoff N, Olney JW. Prolonged exposure to inhalational anesthetic nitrous oxide kills neurons in adult rat brain. *Neuroscience*. 2003;122(3):609-616. doi:10.1016/j.neuroscience.2003.07.012

40. Roberts D, Farahmand P, Wolkin A. Nitrous Oxide Inhalant Use Disorder Preceding Symptoms Concerning for Primary Psychotic Illness. *Am J Addict*. 2020;29(6):525-527. doi:10.1111/ajad.13048

41. Olney JW, Labruyere J, Price MT. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science (80-)*. 1989;244(4910):1360-1362. doi:10.1126/SCIENCE.2660263

42. Sakamoto S, Nakao S, Masuzawa M, et al. The differential effects of nitrous oxide and xenon on extracellular dopamine levels in the rat nucleus accumbens: A microdialysis study. *Anesth Analg*. 2006;103(6):1459-1463. doi:10.1213/01.ane.0000247792.03959.f1

43. Di Chiara G, Bassareo V, Fenu S, et al. Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology*. 2004;47(SUPPL. 1):227-241. doi:10.1016/J.NEUROPHARM.2004.06.032

44. McKendrick G, Graziane NM. Drug-Induced Conditioned Place Preference and Its Practical Use in Substance Use Disorder Research. *Front Behav Neurosci*. 2020;14(September):1-15. doi:10.3389/fnbeh.2020.582147

45. Panlilio L V., Goldberg SR. Self-administration of drugs in animals and humans as a model and an investigative tool. *Addiction*. 2007;102(12):1863. doi:10.1111/J.1360-0443.2007.02011.X

46. Tracy ME, Slavova-Hernandez GG, Shelton KL. Assessment of reinforcement enhancing effects of toluene vapor and nitrous oxide in intracranial self-stimulation. *Psychopharmacology (Berl)*. 2014;231(7):1339-1350. doi:10.1007/s00213-013-3327-y

47. Wood RW, Grubman J, Weiss B. Nitrous oxide self-administration by the squirrel monkey. *J Pharmacol Exp Ther*. 1977;202(3).

48. Walker DJ, Zacny JP. Within- and between-subject variability in the reinforcing and subjective effects of nitrous oxide in healthy volunteers. *Drug Alcohol Depend*. 2001;64(1):85-96. doi:10.1016/S0376-8716(00)00234-9

49. Harper MH, Winter PM, Johnson BH, Koblin DD, Eger IInd EI. Withdrawal convulsions in mice following nitrous oxide. *Anesth Analg*. 1980;59(1):19-21. doi:10.1213/00000539-198001000-00004

50. Kosobud A, Kebabian C, Rebec G. Nitrous oxide acutely suppresses ethanol consumption in HAD and P rats. *Int J Neurosci*. 2006;116(7):835-845. doi:10.1080/00207450600754079

51. Benturquia N, Le Guen S, Canestrelli C, et al. Specific blockade of morphine- and cocaine-induced reinforcing effects in conditioned place preference by nitrous oxide in mice. *Neuroscience*. 2007;149(3):477-486. doi:10.1016/j.neuroscience.2007.08.003

52. Ridenour TA, Halliburton AE, Bray BC. Does DSM-5 Nomenclature for Inhalant Use Disorder Improve Upon DSM-IV? Published online 2014. doi:10.1037/adb0000007

53. Milne B. Nitrous oxide (laughing gas) inhalation as an alternative to electroconvulsive therapy. *Med Hypotheses*. 2010;74(5):780-781. doi:10.1016/j.mehy.2009.11.021

54. Pawson P, Forsyth S. Anesthetic agents. *Small Anim Clin Pharmacol*. Published online January 1, 2008:83-112. doi:10.1016/B978-070202858-8.50007-5

55. Herren MD. Volatile anesthetics. *Anesth Secrets*. Published online 2011:75-81. doi:10.1016/B978-0-323-06524-5.00010-6

56. Bezuidenhout E. The blood – gas partition coefficient. 2020;26:8-11.

57. Piiper J, Scheid P. Blood-gas equilibration in lungs. *Pulm gas Exch*. 1980;1:131-171.

58. Silverthorn DU. Gas exchange and transport. In: *Human Physiology An Integrated Approach*. 7th ed. ; 2016:588-595.

59. Abhang PA, Gawali BW, Mehrotra SC. Technological Basics of EEG Recording and Operation of Apparatus. *Introd to EEG- Speech-Based Emot Recognit*. Published online January 1, 2016:19-50. doi:10.1016/B978-0-12-804490-2.00002-6