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## Rapid antidepressant potential of nitrous oxide: current state and major questions

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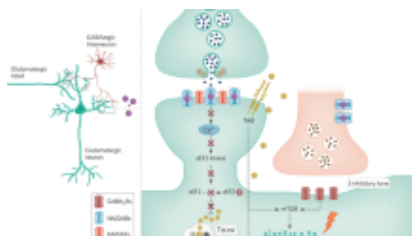
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### Abstract

The success of ketamine, a dissociative anesthetic and non-competitive N-methyl-D-aspartate receptor (NMDAR) antagonist, as a rapidly acting antidepressant has ignited efforts to identify other novel depression treatments. In recent years, several clinical trials indicated that nitrous oxide (N<sub>2</sub>O), an inhalational dissociative anesthetic in clinical use for over 150 years, also has rapid and durable antidepressant effects in patients with major depressive disorder (MDD) and treatment resistant major depression (TRMD). N<sub>2</sub>O is a non-competitive NMDAR inhibitor but acts on NMDARs by mechanisms distinct from ketamine. Cellular and neuronal circuit studies of N<sub>2</sub>O-induced psychotropic and antidepressant effects are in their infancy and suggest that N<sub>2</sub>O shares at least some downstream mechanisms with ketamine, while also having

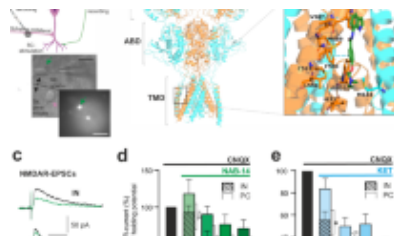
unique effects on neurophysiology and signaling. Human neuroimaging and brain network connectivity studies of N<sub>2</sub>O have begun to identify acute and persisting effects of the drug on brain circuits likely relevant for antidepressant responses. In this review, we highlight the current state of clinical and preclinical research into the effects of N<sub>2</sub>O and emphasize major unanswered questions, some of which are currently being explored. We emphasize future directions and potential barriers to clinical use of N<sub>2</sub>O for treatment of patients with psychiatric illnesses.

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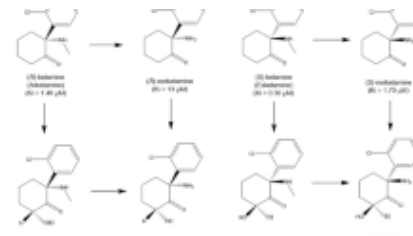
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## Introduction

Psychiatry is witnessing a revolution in therapeutics fueled by the success of ketamine and esketamine as rapidly acting antidepressants [1]. While traditional antidepressants are helpful, they typically have slow onset of action and limited overall efficacy with 30% or more of patients with major depressive disorder (MDD)

failing to respond [2, 3]. By contrast, ketamine produces rapid antidepressant benefits in treatment resistant major depression (TRMD) within hours after a sub-anesthetic intravenous infusion [1]. Intranasal esketamine (the (S+)-ketamine enantiomer) also has rapid antidepressant actions, is FDA approved for TRMD and has an FDA-indication for use in treating depression with suicidal ideation or behavior [1]. Effects of ketamine can persist for days to weeks and outlive the presence of ketamine and its metabolites in brain. Nevertheless, recent meta-analyses indicate that esketamine has significant limitations [4, 5]. Current efforts aim to optimize dose and frequency of ketamine to extend its therapeutic benefits while minimizing its side effects [4].

Based on the success of ketamine and esketamine, there has been interest in identifying alternative pharmacological treatments with rapid onset and longer-lasting improvement, ideally with fewer side effects. Examples of these alternatives include the FDA-approved neuroactive steroids, brexanolone and zuranolone, for postpartum depression [6] and certain experimental agents such as the serotonergic psychedelics including psilocybin [7] and the anti-muscarinic, scopolamine [8]. Additionally, there is interest in repurposing other anesthetic agents based on their rapid modulation of major excitatory (glutamate) and inhibitory (GABA) neurotransmitter systems and the role of these systems in psychiatric illnesses [9,10,11]. In this review, we focus on the inhalational anesthetic, nitrous oxide (N<sub>2</sub>O), and discuss clinical studies in mood disorders and other potential indications. We also discuss mechanistic studies and compare N<sub>2</sub>O to ketamine. We end with a discussion of outstanding questions about N<sub>2</sub>O and its potential use in psychiatry.

## Clinical use of nitrous oxide

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Although there were early anecdotal reports about antidepressant effects of N<sub>2</sub>O [12, 13], the first formal N<sub>2</sub>O clinical trial was conducted following clinical trial successes with ketamine [14]. Ketamine is a non-competitive inhibitor of N-methyl-D-aspartate glutamate receptors (NMDARs) [14], and this mechanism motivated a major change in understanding antidepressant neuroscience. Preclinical studies in the 1990's found that N<sub>2</sub>O is also a non-competitive NMDAR antagonist, although working through receptor mechanisms distinct from ketamine [15, 16]. N<sub>2</sub>O has a long history of safe use in anesthesia and has hypnotic, analgesic and anxiolytic properties over a range of concentrations [17] (Supplemental Fig. 1). Even at high concentrations, N<sub>2</sub>O is a weak anesthetic that is typically used in combination with other anesthetics for general anesthesia or as a sole agent in outpatient dentistry where its dose is limited to 70% or less to avoid hypoxia. N<sub>2</sub>O has relatively mild acute side effects that include nausea and vomiting, dizziness, headaches and euphoria [18, 19]. Some patients experience anxiety and dysphoria during administration. Side effects are usually transient and dissipate rapidly once inhalation is stopped. N<sub>2</sub>O inhibits vitamin B<sub>12</sub> and can cause peripheral neuropathy but concerns about this are mostly relevant to prolonged or chronic use [17]. Emergence of psychosis with N<sub>2</sub>O is rare and typically associated with misuse of high doses of the drug. Dissociative symptoms have been observed with subanesthetic ketamine in healthy volunteers; these symptoms typically occur during infusion and abate within 30-60 min after drug administration is discontinued [20, 21]. Extensive evidence from use of N<sub>2</sub>O in obstetrics, pediatrics, dentistry, and emergency medicine supports its safe use in vulnerable populations. Akin to ketamine, N<sub>2</sub>O has abuse potential, but risks appear lower than ketamine [21, 22]. N<sub>2</sub>O is not scheduled by the U.S. Drug Enforcement Administration (DEA) while ketamine and esketamine are Schedule III and esketamine is subject to an FDA Risk Evaluation and Mitigation Strategy (REMS). Nonetheless, it appears that recreational and abusive N<sub>2</sub>O use has increased over the past 20 years [22]. Contraindications to

N<sub>2</sub>O use include severe pre-existing vitamin B<sub>12</sub> or folate deficiency and conditions with significant air-filled spaces such as middle ear occlusion, pneumothorax, pneumocephalus and bowel obstruction.

Potential advantages of N<sub>2</sub>O include ease of administration, lack of systemic effects, lack of metabolites, and rapid recovery following inhalation in outpatient settings. Unlike ketamine, N<sub>2</sub>O does not have systemic side effects such as elevated blood pressure. It has rapid onset and offset of action, features that reflect its low blood solubility [17]. Most patients recover within minutes after N<sub>2</sub>O inhalation ceases, which allows patients to transport themselves after treatment recovery, in contrast to a minimum 2-hour recovery and alternative transportation required by REMS for esketamine. Because most of the inhaled N<sub>2</sub>O is exhaled, it has minimal interactions with other drugs and has no metabolites, so once the drug is cleared after inhalation further direct drug action is not a factor. This latter point has important clinical implications and relevance for understanding the mechanisms underlying psychotropic effects - namely that all direct drug effects leading to persisting changes are initiated during drug administration. This contrasts with ketamine, which has a several-hour half-life and produces active metabolites, such as 2,6-hydroxynorketamine and norketamine, which may exert effects beyond the initial dosing period, based on preclinical studies [23,24,25].

## Current state of nitrous oxide in psychiatry

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As noted, N<sub>2</sub>O has a long history in anesthesiology and there is more recent interest in its potential use in neuropsychiatry [13]. The first modern trial of N<sub>2</sub>O for TRMD was reported a decade ago and seven trials for depression have been published to date, most involving subjects with TRMD of varying degrees of refractoriness. The initial

trial [26] was a double-blind, placebo-controlled, crossover study of 20 patients with severe TRMD who had failed an average of 8 adequate dose-duration antidepressant trials including 4 individuals who had failed electroconvulsive therapy and 3 who had failed vagus nerve stimulation. Patients were treated for one hour with 50% N<sub>2</sub>O or placebo as tolerated. Four subjects showed full response (>50% reduction in depressive symptoms) at the primary endpoint twenty-four hours after N<sub>2</sub>O treatment, and three met clinical criteria for remission; only one individual responded to placebo. By design, the crossover between N<sub>2</sub>O and placebo occurred one week after treatment. Many who experienced N<sub>2</sub>O antidepressant benefit showed carryover antidepressant effects at that timepoint, suggesting that a subset of TRMD patients experience sustained antidepressant response beyond one week following a single N<sub>2</sub>O administration. Five subjects had N<sub>2</sub>O inhalation terminated before one hour because of side effects including nausea, headache and emotional discomfort, while no patients receiving placebo (oxygen + air) had early termination.

This initial trial was followed by a dose-finding study comparing 25% and 50% inhaled N<sub>2</sub>O for one hour vs. placebo (oxygen + air), based on the premise that a lower concentration of N<sub>2</sub>O that still inhibits NMDARs [15, 16] would be effective with potentially minimized side effects. This study was also a double-blind, randomized, crossover design with switches occurring at 4-weeks after N<sub>2</sub>O or placebo [27]. In 24 subjects with moderate to severe TRMD (4-5 treatment failures on average) both 25% and 50% N<sub>2</sub>O improved depressive symptoms with no apparent antidepressant efficacy difference between the two doses. At the time of crossover, a subset of subjects who received N<sub>2</sub>O first remained improved while placebo-treated patients had returned to their depressed baseline state. Additionally, the 25% N<sub>2</sub>O dose demonstrated superior tolerability, with a four-fold lower risk of side effects.

Several other studies have examined N<sub>2</sub>O effects in TRMD [28]. In a double blind,

placebo-controlled trial, Yan et al. [29] studied 20 TRMD participants receiving 50% N<sub>2</sub>O for one hour compared to 24 placebo-treated TRMD patients. Participants had experienced 2 or more treatment failures in the current episode. Depressive symptoms improved at 2 and 24 h after N<sub>2</sub>O but did not differ from placebo at one or two weeks. N<sub>2</sub>O-treated subjects did show improved executive function one week after N<sub>2</sub>O [30]. In an open label trial, Desmidt et al. [31] studied 20 women with mild TRMD (one treatment failure) using 50% N<sub>2</sub>O for one hour and found 25% and 45% clinical response at 24 h and 7 days, respectively. Kim et al. [32] reported improvement in 12 participants with bipolar disorder and treatment resistant depression (vs. 13 controls) in a double-blind, placebo-controlled trial using a final dose of 25% N<sub>2</sub>O for 20 min. These subjects showed improved depression on the day of treatment but not at 24 h.

Ladha et al. recently described a randomized, double blind, parallel design, pilot trial comparing 50% N<sub>2</sub>O-50% O<sub>2</sub> plus intravenous saline infusion (N=20) to active placebo with intravenous midazolam and 50% O<sub>2</sub> (N=20) in participants with TRMD and two treatment failures in the current episode [33]. N<sub>2</sub>O and placebo were administered for one hour once weekly for four weeks. At day 42 follow up (3 weeks

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group achieved a clinical response defined as >50% change in depression score while 16.7% of those receiving N<sub>2</sub>O showed a response (5.6% achieved clinical remission). Adverse effects included nausea and vomiting, headache and anxiety/chest tightness and were described as mild to moderate and transient but more frequent in subjects receiving N<sub>2</sub>O. One participant receiving N<sub>2</sub>O required hospitalization for worsening mood symptoms.

Two clinical trials to date have examined N<sub>2</sub>O for MDD in non-TRMD subjects. Guimaraes et al. [34] conducted a double-blind, parallel design trial of 12 N<sub>2</sub>O and 11 placebo-treated individuals using 50% N<sub>2</sub>O administered for one hour twice weekly for four weeks. Subjects receiving N<sub>2</sub>O showed 92% response and 75% remission after four weeks of treatment. Myles et al. [30] studied 81 patients with MDD treated with either 25% or 50% N<sub>2</sub>O for 1 h versus 41 with placebo (air/oxygen) administered once weekly for four weeks in a randomized, double-blind trial. MDD subjects in this trial included individuals with TRMD with a median of 6 treatment failures. Although recruitment was limited by the pandemic and the study failed to meet its primary endpoint at 4 weeks, the study met its secondary endpoint and N<sub>2</sub>O resulted in 38% of participants exhibiting remission by 1 week compared to 13% of controls. Side effects included light-headedness and nausea and were greater in the group receiving 50% N<sub>2</sub>O.

In summary, several small placebo-controlled studies have reported benefits of N<sub>2</sub>O in patients with TRMD and MDD (summarized in Table 1). In most studies, benefits were manifest prior to or by 24 h after inhalation and persisted for up to several weeks in some participants. Limitations and challenges in interpreting these studies and potential strategies for improving study designs are discussed below.

### **Table 1 Clinical Trials of Nitrous Oxide in TRMD & MDD.**

## **Mechanistic studies of nitrous oxide**

### Effects on human brain networks

There is limited information about the effects of N<sub>2</sub>O on human brain networks in

either healthy or depressed individuals, especially effects persisting beyond the time of drug administration. In healthy subjects, Dai and colleagues [35, 36] examined acute “psychedelic-like” effects (altered consciousness) of 35% N<sub>2</sub>O for 40 min. Using functional magnetic resonance imaging (fMRI), they observed lower functional connectivity within several brain networks but increased functional connectivity between networks. Notable changes were observed between the temporo-parietal junction and bilateral intraparietal sulci and between the precuneus in the default mode network (DMN) and the left intraparietal sulcus. Other changes included decreased functional differentiation in the frontoparietal and somatomotor networks, with overall flattening of cortical geometry and disruption of network temporal dynamics. Several identified areas, including the DMN, are important for affective processing and have previously been linked to depression [37]. Interestingly, the acute human neuroimaging effects of N<sub>2</sub>O on within and between network functional connectivity in the Dai et al. studies [35, 36] were similar to what this group also observed with ketamine and LSD despite the drugs having distinctly different molecular actions.

In 16 healthy volunteers, Palanca et al. [38] observed sustained functional connectivity changes induced by one hour exposure to 50% N<sub>2</sub>O compared to air-oxygen administration. Linearly increasing changes in global functional connectivity within occipital cortex and between visual cortex and the dorsal attention network were observed at 2 and 24 h after N<sub>2</sub>O exposure. Weaker changes were observed between visual cortex and the frontoparietal (attention) network and DMN. These results demonstrate that the effects of N<sub>2</sub>O on human brain networks can persist at least 24 h following a single one-hour inhalation but leave unanswered whether these changes relate to effects in depressed patients.

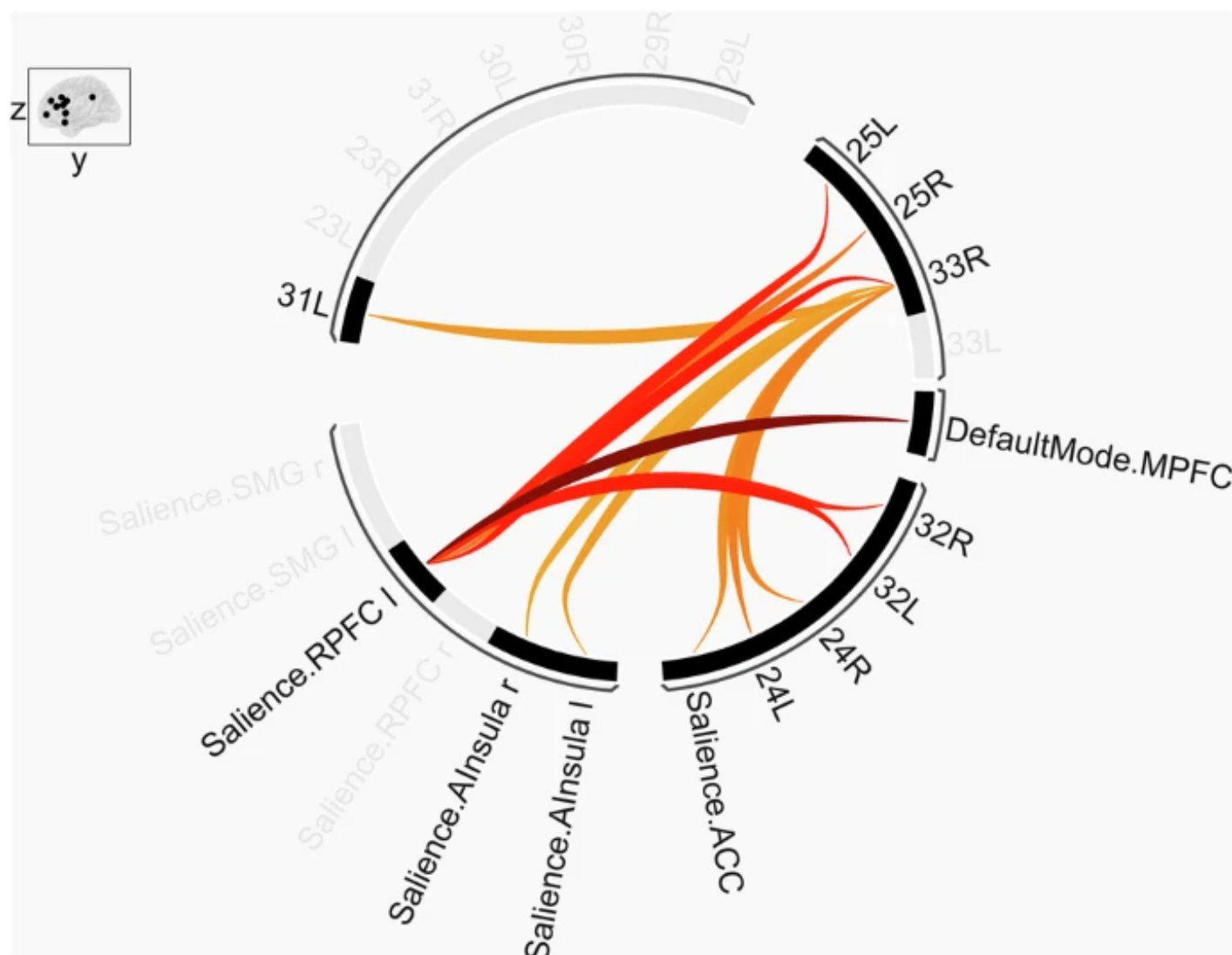
One fMRI study has examined functional connectivity changes in depressed

participants treated with N<sub>2</sub>O. Desmidt et al. [31] found decreased functional connectivity 2-hours after a 1h 50% N<sub>2</sub>O exposure between the subgenual anterior cingulate cortex (sgACC) and precuneus in women with mild TRMD who had a clinical response to N<sub>2</sub>O. In contrast, they observed diminished connectivity between the supracallosal ACC and mid-cingulate cortex in healthy controls and in depressed subjects who failed to respond to N<sub>2</sub>O exposure. This study also included ultrasound measurements consistent with increased regional cerebral blood flow (CBF). Using arterial spin labelling MRI, Kim et al. [32] observed that lower pre-N<sub>2</sub>O cerebral blood flow predicted greater N<sub>2</sub>O response at 24 h in a study of patients with bipolar disorder and refractory depression treated with up to 25% N<sub>2</sub>O.

A recent human imaging study published in preprint form [39] used a randomized cross-over design with 50% N<sub>2</sub>O vs. placebo exposure for one hour with treatments spaced one month apart. This study examined functional connectivity changes in five limbic seeds in 14 TRMD and 16 non-depressed participants at baseline and 2- and 24-hours after N<sub>2</sub>O exposure. The seeds probed networks that participate in MDD/TRMD (DMN, salience, reward, and cingulo-opercular networks). Over 24 h, N<sub>2</sub>O progressively decreased connectivity in all depression-related networks in subjects with TRMD while connectivity was increased in those same networks in non-depressed controls. Functional connectivity changes were also observed in the dorsal paracingulate cortex, a region known as a “dorsal nexus” that shows hyperconnectivity in MDD [40] (Fig. 1). These N<sub>2</sub>O-induced functional connectivity changes were not mimicked by placebo. This study supports the idea that N<sub>2</sub>O persistently reduces functional connectivity beyond 2 h in limbic regions in TRMD and that the effects are related to the presence of depressive illness. The functional connectivity changes moved in opposite directions for the two groups, with N<sub>2</sub>O-associated decreases in functional connectivity in TRMD and increases in non-

depressed controls. These state-dependent (MDD-associated) functional connectivity changes are akin to those observed with ketamine [41].

**Fig. 1: Medial limbic structures exhibit higher functional connectivity in untreated TRMD patients compared to non-depressed controls.**



The connectome ring display shows higher functional connectivity among cingulate cortex seeds (subgenual (Brodmann Area (BA) 25), anterior cingulate (BA33), ventral anterior cingulate (BA24), dorsal anterior cingulate (BA32), dorsal posterior cingulate (BA31)) and the salience and default mode networks. Broader connections between seed paths show greater between-group differences. Path maximum t-value = 4.08.

Two studies have examined effects of N<sub>2</sub>O exposure on electroencephalographic (EEG) measures of brain activity and connectivity in depressed subjects. In patients with TRMD, Shao et al. [42] reported increases in brain connectivity that correlated with changes in depressive symptoms 24 h after N<sub>2</sub>O. They also observed changes in task-based event related potentials (ERPs) using a face recognition task. In three subjects, Kronenberg et al. [43] observed increased alpha power along with right frontal alpha asymmetry and an overall decrease in EEG vigilance in depressed individuals who responded to N<sub>2</sub>O treatment.

While data are limited, these fMRI and EEG studies provide tentative support for the idea that N<sub>2</sub>O has detectable effects on human brain functional connectivity in networks contributing to cognition and emotion. Specific networks studied include the DMN (self-referential thinking), salience and reward networks (emotional valance and motivation) and the cingulo-opercular cognitive control network (error monitoring and alertness) [44, 45]. To date, changes in these networks have not been linked to specific depressive symptom domains following N<sub>2</sub>O; however, studies are currently underway. Some MDD-specific effects of N<sub>2</sub>O exposure (reducing connectivity in TRMD while increasing connectivity in controls), have been observed in ketamine neuroimaging studies [41]. There is less information about persisting effects of N<sub>2</sub>O exposure, but recent studies in TRMD and healthy controls show connectivity changes that last at least 24 h after drug [38, 39]. As noted earlier, clinical TRMD studies have demonstrated sustained mood effects beyond 24 h [26, 27]; whether functional connectivity changes persist beyond 24 h remains to be clarified.

Receptor, ion channel & neural circuitry effects in preclinical studies

N<sub>2</sub>O is a non-competitive NMDAR antagonist [15], acting by mechanisms distinct from ketamine. While ketamine inhibits open NMDAR channels through sites within the ion channel pore and results in a use- and voltage-dependent form of block, effects of N<sub>2</sub>O are neither strongly voltage- nor use-dependent. Consistent with this, ketamine speeds decay of NMDAR-gated currents including excitatory postsynaptic currents, a feature not shared by N<sub>2</sub>O [16]. It is presently unknown where or how N<sub>2</sub>O acts within the NMDAR complex, and unlike ketamine there is little information about effects on NMDARs of varied subunit composition. However, certain behavioral changes induced by N<sub>2</sub>O have been linked to effects on NMDARs [46].

N<sub>2</sub>O also weakly inhibits the AMPA class of glutamate receptors and weakly potentiates inhibitory GABA<sub>A</sub>Rs [16]. The latter effect may contribute to anxiolysis and may be mediated by the benzodiazepine binding site on GABA<sub>A</sub>Rs [47]. The drug also has weak effects on GABA receptors comprised of rho subunits (sometimes called GABA<sub>C</sub>Rs) [48]. N<sub>2</sub>O is a partial inhibitor of low voltage activated, Cav3.2 but not Cav3.1, T-type Ca<sup>2+</sup> channels [49]. T-channel effects do not appear to be shared by ketamine. Because T-currents and NMDARs contribute to behaviorally important burst firing in lateral habenula, inhibition of T-currents by N<sub>2</sub>O and/or NMDAR antagonism (both N<sub>2</sub>O and ketamine) could suppress this burst firing and contribute to antidepressant-like changes [50, 51]. The lateral habenula, a key relay linking limbic regions with midbrain monoaminergic systems, encodes negative valence signals. Aberrant activity in this region participates in depressive-like symptoms in preclinical studies, including aversive and anhedonia-like behaviors [52,53,54]. Human studies provide mixed support for findings in habenula in mood disorders but indicate modulation by ketamine [55, 56].

N<sub>2</sub>O activates α-adrenergic receptors [57, 58] and weakly inhibits serotonin-3 (5HT<sub>3</sub>) and certain nicotinic acetylcholine receptors (α4β2 and α4β4) [48]. The drug also

activates 2-pore domain TREK-1 potassium channels [59]. An intriguing effect that could contribute to psychiatric and analgesic properties is N<sub>2</sub>O's ability to stimulate certain opioid receptors [60]. Opioid receptor effects of N<sub>2</sub>O preferentially involve κ-opioid receptors with lesser contribution from δ-receptors [61, 62] and are observed at concentrations used to treat depression, and that affect NMDARs [63,64,65,66]. In rats, N<sub>2</sub>O produces acute analgesic effects via opioid receptors. However, a single administration of 50% N<sub>2</sub>O for 75 min can dampen neuropathic pain in a chronic sciatic nerve injury model for several weeks by mechanisms independent of acute opioid receptor effects and possibly involving NMDAR inhibition [67]. Effects of ketamine on the opioid system have also been described, and μ-type opioid receptors may contribute to antidepressant activity in humans [4] and changes in glutamate signaling based on magnetic resonance spectroscopy measures [68]. Preclinical studies with ketamine suggest that the opioid system is more likely permissive and not sufficient for antidepressant-like changes [69]. The role of the endogenous opioid system in the effects of ketamine in concert with NMDARs remains an important area of active investigation [70].

Mechanistic preclinical studies of N<sub>2</sub>O on rodent brain circuits are limited and have been informed by work on ketamine. Despite its NMDAR antagonism and the role that NMDAR activation plays in triggering synaptic plasticity, ketamine persistently enhances glutamatergic transmission in several brain regions [71,72,73]. This synaptic enhancement occurs rapidly and involves increased expression of synaptic AMPARs and structural changes in dendrites [71]. Potentiating effects of ketamine involve several intracellular signaling systems including mechanistic target of rapamycin (mTOR) [71], brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) [72,73,74,75], and nitric oxide synthase (NOS) [76,77,78]. While effects on nitric oxide (NO) synthesis have been observed in

preclinical studies, it is not clear how these effects translate to antidepressant actions in humans based on a study using sodium nitroprusside as a NO donor [79].

Less is known about effects of N<sub>2</sub>O on circuit function, but several findings are consistent with ketamine's effects. In adult rat hippocampal slices, acute perfusion of 30% N<sub>2</sub>O causes initial depression of AMPAR-mediated synaptic responses during drug administration (unlike ketamine) [78]. This initial depression may reflect changes in tissue oxygenation, weak inhibition of AMPARs [16] and/or presynaptic effects [80, 81]. Following N<sub>2</sub>O perfusion, there is a rapid and persistent enhancement of glutamate transmission that affects both AMPAR and NMDAR synaptic responses. N<sub>2</sub>O engages similar signaling systems as ketamine. Early events include activation of NMDARs that remain unblocked during acute drug administration. Thus, complete NMDAR inhibition with a high concentration of a standard competitive NMDAR antagonist during N<sub>2</sub>O administration prevents synaptic enhancement [71, 78, 82]. Other shared mechanisms include mTOR, NOS and TrkB receptors [78, 83]. A key question concerns how ketamine and N<sub>2</sub>O activate unblocked NMDARs. For ketamine, this appears to involve local disinhibition in which ketamine preferentially inhibits NMDARs on interneurons, possibly NMDARs expressing GluN2B or GluN2C/D subunits [84,85,86,87]. Akin to ketamine, N<sub>2</sub>O disinhibits the CA1 hippocampal circuit contributing to changes in local excitation [88]. A potential mechanism contributing to disinhibition is the ability of N<sub>2</sub>O to weakly enhance synaptic GABA<sub>A</sub> receptor inhibition of hippocampal interneurons [16]. A challenge in relating these effects of N<sub>2</sub>O on CA1 transmission to antidepressant actions is that these studies were done in naïve (unstressed) rodents. Thus, the observed enhanced transmission may mirror the increased functional connectivity observed in healthy human controls after N<sub>2</sub>O exposure [38, 39], in contrast to the diminished connectivity in mood-related brain circuits observed in depressed subjects [31, 39].

In other preclinical studies, N<sub>2</sub>O increases expression of the *bdnf* gene and immediate early genes *cFos* and *Arc* while promoting phosphorylation of TrkB receptors [89]. Transcriptional changes occur acutely and are followed by phosphorylation of TrkB, glycogen synthase kinase 3 and mitogen activated protein kinase (MAPK). These phosphorylation events occur after N<sub>2</sub>O exposure at a time when cortical EEGs show rebound homeostatic slow waves. A recent study in mice linked post-N<sub>2</sub>O-induced changes in TrkB activation, EEG oscillations, glucose utilization and antidepressant-like effects to decreases in body temperature [90]. N<sub>2</sub>O exposure also rapidly increases calcium signals in mPFC that precede changes in cortical EEG activity detected in both sleep (increased slow wave activity) and awake states (increased gamma oscillations) ([91], preprint). In healthy human participants (N=7 men), 50% N<sub>2</sub>O for 20 min increased EEG gamma power during administration with decreases in alpha and beta band power. In the 10 min following N<sub>2</sub>O, increases in gamma oscillations persisted while increases in theta but not delta frequency power were also observed, differing from observations in mice [92]. Other evidence indicates that N<sub>2</sub>O activates NOS early, promoting BDNF expression, antidepressant-like activity [93] and possibly anxiolytic-like effects [94]. Changes in nitric oxide could also contribute to the effects on body temperature noted above [90]. The time course of these latter effects may differ from those of ketamine because ketamine stimulates early translation of the *bdnf* gene followed by subsequent transcriptional changes [87].

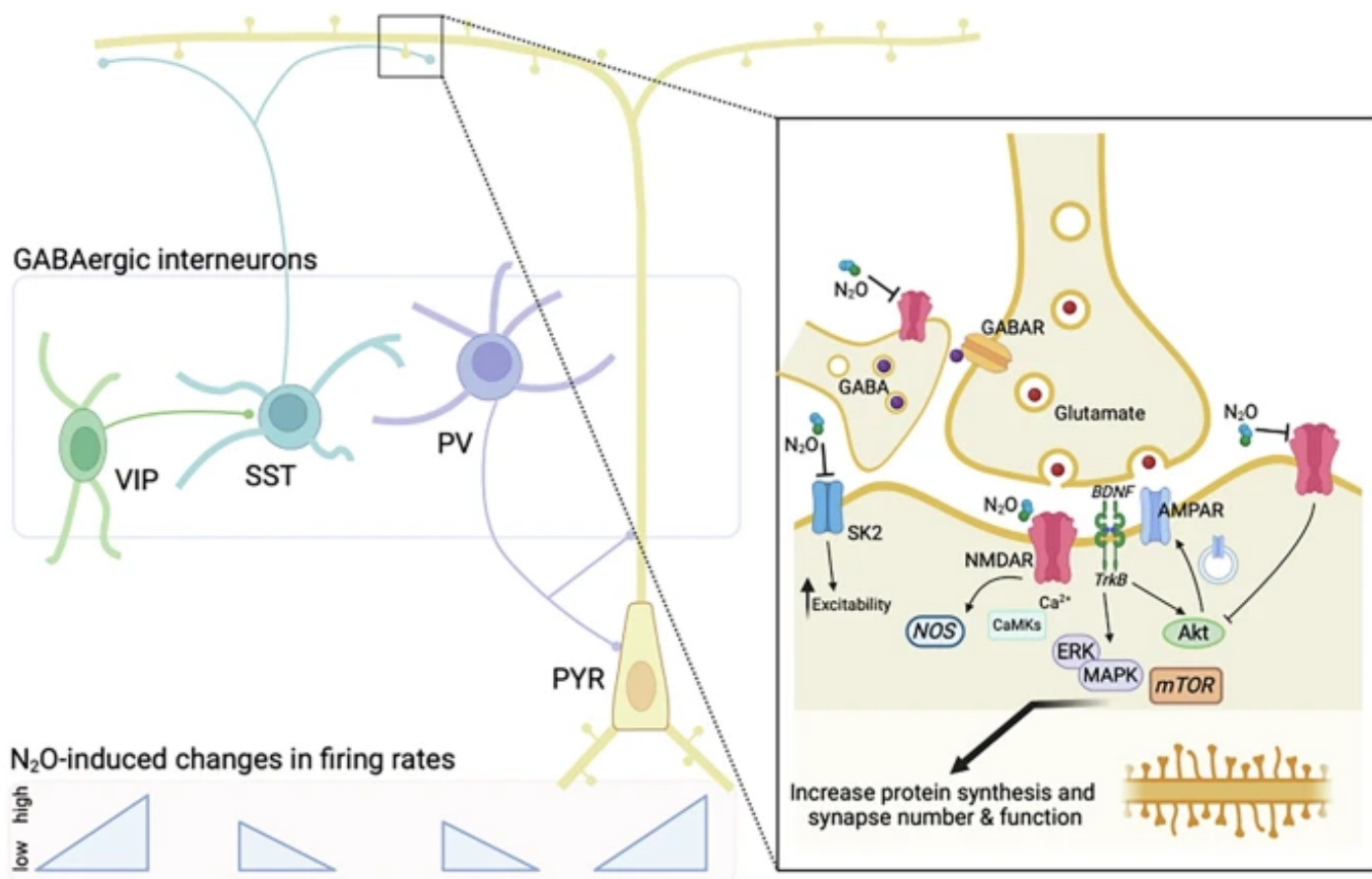
Hippocampal synaptic enhancement by N<sub>2</sub>O occludes potentiation by ketamine, supporting overlapping mechanisms. However, LTP induced by electrical stimulation of hippocampal synapses remains intact after N<sub>2</sub>O administration [78]. AMPARs play a key role in synaptic potentiation by ketamine, acting both as a trigger for enhancement and as a mediator of potentiation via increased receptor expression [95]. In contrast, induction of synaptic enhancement by N<sub>2</sub>O does not require AMPAR

activation [78]. This latter finding likely reflects differences in how the drugs inhibit NMDARs. With ketamine, NMDAR channels must open for block to occur. Hence, complete block of AMPARs dampens the effects of ketamine on NMDAR channels by preventing AMPAR-mediated depolarization. N<sub>2</sub>O does not require channel opening to inhibit NMDARs [16]. Another important difference is that N<sub>2</sub>O, unlike ketamine, is cleared rapidly once administration ends and has no active metabolites.

A recent preclinical study in medial prefrontal cortex (mPFC) further elucidates N<sub>2</sub>O's cellular and circuit mechanisms, highlighting a potentially novel effect that may differ from ketamine [96]. In this study, 50% N<sub>2</sub>O for one hour produced rapid activation of layer 5 (L5) pyramidal neurons in wild-type, chronically stressed mice. This L5 activation reversed stress-induced hypoactivity in L5 and was associated with antidepressant-like behavioral effects. Enhanced L5 activity involved disinhibition via effects on somatostatin- and parvalbumin-expressing interneurons resulting from activation of interneurons expressing vasoactive intestinal peptide (VIP) (Fig. 2). Importantly, L5 activation involved inhibition of SK2 potassium channels rather than NMDARs; both L5 pyramidal neurons and VIP interneurons express SK2 channels. SK2 channels contribute to the medium afterhyperpolarization (AHP) that follows action potential firing and help to regulate neuronal excitability [97,98,99]. While NMDARs did not directly trigger L5 changes, NMDARs contributed to persistent activity changes following N<sub>2</sub>O exposure. Importantly, this study raises questions about the primary role of NMDARs in antidepressant-like effects triggered in mPFC. Similarly, there are remaining questions about the role of NMDARs in the effects of ketamine considering metabolites that have antidepressant-like properties but marginal NMDAR effects based on preclinical studies [1, 25, 100].

**Fig. 2: The scheme depicts mechanisms contributing to effects of N<sub>2</sub>O in**

## hippocampus and mPFC.



In hippocampus, N<sub>2</sub>O disinhibits the CA1 circuit likely by inhibiting NMDARs on GABAergic interneurons, resulting in increased activity of pyramidal (glutamatergic) neurons (Fig. 2). This triggers activation of unblocked postsynaptic NMDARs and stimulates downstream signaling involving BDNF, NOS and mTOR, along with MAPK, ERK and Akt. In mPFC, inhibition of SK2 channels on VIP interneurons triggers disinhibition via somatostatin (SST) and parvalbumin (PV)-expressing interneurons, while inhibition of SK2 on L5 pyramidal neurons contributes to increased excitability. The net result is persistently enhanced excitatory function. This figure was made with BioRender.

Also in PFC, N<sub>2</sub>O and ketamine promote transcription of multiple genes, including dual function phosphatases that regulate MAPKs [101]. In this study, N<sub>2</sub>O produced

more widespread effects on gene expression, while ketamine, but not N<sub>2</sub>O, increased spiking of pyramidal neurons and increased gamma frequency oscillations. In other rodent studies, 50% N<sub>2</sub>O transiently increases EEG signal complexity and high gamma band power associated with increased acetylcholine (ACh) levels in prefrontal and parietal regions. These early changes last about 12 min and are followed by reduced complexity, weaker frontoparietal high gamma band power and lower ACh levels over a 1-hour drug exposure [102]. Effects of N<sub>2</sub>O differed from subanesthetic ketamine, which showed sustained increases in these parameters over the period of drug exposure. Again, these findings suggest potentially important differences between N<sub>2</sub>O and ketamine that remain to be clarified and fully understood (Table 2).

### **Table 2 Nitrous oxide & ketamine: cellular & synaptic mechanisms.**

In a preclinical study in mice using a drug administration paradigm designed to mimic repeated abusive drug use (20% N<sub>2</sub>O daily for 4 consecutive days), N<sub>2</sub>O was found to upregulate transmission in the ventral tegmental area-nucleus accumbens (VTA-NAc) reward pathway by effects on BDNF and D1 dopamine receptor activity [103]. Whether similar changes occur in this reward/motivation circuit with N<sub>2</sub>O dosing used for depression is unknown. In recent studies, ketamine has been shown to exert anti-anhedonia-like effects via VTA-NAc signaling [73].

## **Key questions about nitrous oxide**

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### Clinical questions

The studies outlined in this review highlight ongoing progress in understanding N<sub>2</sub>O as a potential psychiatric treatment. It is clear, however, that there are presently more

questions than answers. Here we will enumerate some of the major issues and potential solutions.

1. Clinical evidence supporting antidepressant effects of N<sub>2</sub>O is based on a small number of trials with relatively few participants. Thus, there is major need for larger, multi-site, double-blind, placebo-controlled trials potentially allowing analysis of clinical subgroups of patients, including non-resistant MDD, bipolar depression (including bipolar patients with treatment resistant depression) and unipolar TRMD. To date, there is little information about effects of N<sub>2</sub>O in these latter individuals. Further work on N<sub>2</sub>O dosing is needed to determine whether fixed, titrated, or multidose schedules are most effective and if titration helps to minimize side effects [104]. The duration and magnitude of N<sub>2</sub>O exposure can be much more easily controlled compared to ketamine, perhaps permitting more individualized treatment protocols for specific patients.
2. Antidepressant benefits of N<sub>2</sub>O exposure can be sustained for weeks or longer. This durability varies among participants and complicates studies using crossover designs comparing N<sub>2</sub>O and placebo exposure as there are persisting effects if crossover is scheduled too early. In trials to date some patients show persisting effects of N<sub>2</sub>O a month or longer following a single administration. To avoid crossover complications parallel study designs are critical for future studies.
3. More information is needed about the duration of mood effects. As noted, some improvements in depression can last a month or more. However, this phenomenon has not been studied systematically. Effects of N<sub>2</sub>O likely fade over time, as they do with ketamine. Thus, another challenge is to identify strategies to prolong the therapeutic benefits, by optimizing dose size and frequency. With

ketamine, it appears that repeated administrations, adjunctive rapamycin or the addition of concomitant psychotherapy may help [4]. A major trend in studies of psychedelic treatments is the concomitant use of psychotherapies with psychedelics. Studies suggest that combined treatments may provide better outcomes and support to patients receiving agents that cause dissociation [105, 106]. Most psychedelic studies to date employ psychotherapy, so whether this is essential is still debated [107]. However, this combined treatment model could be an opportunity in N<sub>2</sub>O therapy. A recent preclinical study indicates that inhibition of dual-specificity phosphatase 6 (DUSP6) increases the activity of extracellular signal-related kinase (ERK) downstream to BDNF and enhances synaptic effects of ketamine in hippocampus while prolonging antidepressant-like effects for up to 2 months [108]. Similar effects in humans would be clinically important and likely have relevance for N<sub>2</sub>O given prior work showing upregulation of DUSPs by N<sub>2</sub>O in rodents [101].

4. Multiple clinical trials have used placebo control, typically with other gases (air + oxygen). It is unclear whether this placebo is satisfactory and how well blinding is maintained. Recent ketamine trials have used intravenous midazolam as a control. Midazolam or a psychoactive variant could be considered in N<sub>2</sub>O trials as an active control for sedating effects of the drug. A recent pilot trial used midazolam as a placebo and showed beneficial effects of N<sub>2</sub>O [33]. Alternatively, low doses of N<sub>2</sub>O (10% or less) could be explored to determine a minimum effective antidepressant dose. Exploring additional N<sub>2</sub>O doses could also help characterize side effects in psychiatric settings. We note that in studies to date the use of air + oxygen placebo has been associated with significant placebo responses that appear to dissipate by two weeks after inhalation [27].

5. Other extant questions concern whether N<sub>2</sub>O shares ketamine's apparent

beneficial effects on suicidality and the possible use of N<sub>2</sub>O in other psychiatric disorders. To date, only one secondary analysis [109] has described effects of N<sub>2</sub>O on suicidal ideation and provides tentative support for possible anti-suicidal effects. Systematic studies examining N<sub>2</sub>O for psychiatric indications other than depression are lacking. A small open label study [110] found that two of three patients with severe post-traumatic stress disorder (PTSD) responded to 50% N<sub>2</sub>O. This observation is consistent with an experimental study reporting that N<sub>2</sub>O exposure can speed reduction of intrusive memories [111]. N<sub>2</sub>O administered at 50% for 30 min may also interfere with memory reconsolidation [112] as has been observed in a study of alcohol-related memories in subjects with hazardous drinking [113]. These areas require further exploration in larger, well-designed studies. N<sub>2</sub>O should also be studied in primary anxiety disorders, given its reported anxiolytic properties [92, 114, 115, 116]. There are presently multiple trials listed in ClinicalTrials.gov examining N<sub>2</sub>O for treatment of MDD, bipolar depression, PTSD, obsessive compulsive disorder and acute suicidal ideation. Results from these trials should inform future studies.

## Mechanistic questions

There are numerous unanswered questions about how N<sub>2</sub>O works as an anesthetic, analgesic and pharmacotherapeutic across different concentrations and exposure durations and frequency. Here we enumerate several opportunities.

1. Mechanisms underlying N<sub>2</sub>O effects on NMDARs remain uncertain and it is unknown whether N<sub>2</sub>O has preferential actions on NMDAR subtypes as reported for ketamine [24]. N<sub>2</sub>O-mediated NMDAR antagonism differs from ketamine and N<sub>2</sub>O is not an open channel blocker. Thus, direct mechanistic comparisons to ketamine are limited. Based on studies examining N<sub>2</sub>O block of T-type calcium

channels, it is possible that N<sub>2</sub>O acts on NMDARs via a form of redox modulation [117,118,119].

2. N<sub>2</sub>O, like ketamine, persistently enhances excitatory transmission in hippocampus and mPFC, and local circuit disinhibition contributes to these effects. Although partial inhibition of NMDARs triggers synaptic enhancement in hippocampus [78], this effect is less clear in cortex, where SK2 channels play a role [96]. Understanding other regional mechanistic differences will also be important [120]. For ketamine, the role of NMDAR inhibition as an upstream trigger of antidepressant-like effects has been questioned based on the effects of NMDAR-inactive metabolites [25]. Other work has linked persistent blocking effects of ketamine on NMDAR channels to antidepressant-like effects [100]. N<sub>2</sub>O does not appear to have persisting ion channel effects and drug metabolites are unlikely to be involved in its actions. Likewise, mechanistic triggers for N<sub>2</sub>O other than NMDARs have been proposed [60, 96]. Although some data favor a common upstream NMDAR trigger [78], mechanisms of ketamine's NMDAR-inactive metabolites may converge with those of N<sub>2</sub>O downstream.
3. Further work on downstream messengers and changes in neuronal structure and gene expression is needed to understand the drug's persistent effects. N<sub>2</sub>O increases cell proliferation (neurogenesis) in the dentate gyrus. This effect could contribute to slower developing and longer-lived structural plasticity [121]. It is presently unclear how or whether enhancement of excitatory transmission contributes to N<sub>2</sub>O's behavioral and therapeutic effects, although there is tentative support for enhanced synaptic efficacy contributing to ketamine's effects [24, 122,123,124,125]. It is also important to understand whether mechanisms underlying dissociative effects of N<sub>2</sub>O and ketamine contribute to their therapeutic results [126].

4. There are substantial challenges in studying N<sub>2</sub>O in preclinical settings. N<sub>2</sub>O can be difficult to administer, particularly in ex vivo tissue slices where changes in oxygenation can confound results as N<sub>2</sub>O concentrations are increased. On the other hand, N<sub>2</sub>O can be administered in vivo at relevant subanesthetic doses, which permits studies of network functional changes.
5. There is limited information about persisting effects of N<sub>2</sub>O on human brain networks. Preliminary work suggests that N<sub>2</sub>O-associated network changes can be subtle and may require large sample sizes to accurately identify. These efforts would benefit from use of precision functional mapping (PFM) methods to better define changes in individual subjects relevant to therapeutic and adverse effects [127, 128]. It is intriguing that imaging studies to date suggest that N<sub>2</sub>O may have differential persisting circuit effects in healthy controls (increased connectivity) compared to TRMD subjects (diminished connectivity) [31, 38, 39]. Studies of brain oscillations including measures of criticality and stabilization could provide additional insights into mechanisms, given well-characterized effects of ketamine on brain rhythms [129,130,131] and preclinical studies indicating that N<sub>2</sub>O increases slow wave activity following inhalation [89,90,91,92]. N<sub>2</sub>O may also acutely increase beta- and gamma-band EEG power [132].

## Summary & conclusions

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Although these are early days for psychiatric use of N<sub>2</sub>O, accumulating data tentatively support N<sub>2</sub>O as having efficacy for depressive disorders. To date, most studies involve TRMD, and this population may prove to be a niche for the drug, akin to ketamine. Whether N<sub>2</sub>O is as effective as ketamine in TRMD remains to be

determined. Mechanistic studies of N<sub>2</sub>O and ketamine are progressing and indicate both shared mechanisms and some unique effects. It is increasingly clear that traditional anesthetics are altering the therapeutic landscape in psychiatry [133]. Ketamine is the leading example, but N<sub>2</sub>O merits further consideration. Also, certain GABAergic neurosteroids such as allopregnanolone (brexanolone) are endogenous modulators that have served as templates for some anesthetics [134] and have efficacy in neuropsychiatry [9, 135, 136]. Other GABAergic anesthetics, including propofol and isoflurane, are being considered [10, 11, 133].

If larger clinical trials support therapeutic benefits, it will be instructive to see how N<sub>2</sub>O fits into psychiatry. N<sub>2</sub>O is relatively easy to administer and has a long history of safe sub-anesthetic clinical use, especially in outpatient dentistry. It is conceivable that N<sub>2</sub>O could be administered in sub-anesthetic doses in psychiatric outpatient practices, although there will likely be regulatory requirements for its use as in dentistry where practitioners must take a certification course [17]. In hospital settings, N<sub>2</sub>O often falls under the purview of anesthesiology and sedation committees. These features may limit availability for use by psychiatrists but are not insurmountable given the practical ease of drug administration. Lack of FDA approval for the repurposed drug could also be a deterrent as it is with ketamine [137]. Improvements in medical devices and methods for gas delivery are opportunities to enhance patient tolerability and acceptance during inhalation.

The advent of rapidly acting antidepressant treatments with novel mechanisms is providing new options for patients. Many important questions remain [4], however, including the sustainability of effects and risks and benefits of longer-term and repeated use (potentially "perpetual use") of these agents as is being studied with esketamine [138]. How future practice adopts N<sub>2</sub>O will be determined by its effectiveness, duration of effects, and ease of use, relative to its side effects, safety,

risks and cost.

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### Contributions

CFZ conceptualized the paper and wrote the first draft of the manuscript. All other authors (JC, YI, TZ, SM, PN and CRC) contributed to the concepts and content of the article and to editing and approving the final draft. TZ also designed Fig. [1](#) and JC designed Fig. [2](#).

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## Ethics declarations

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Competing interests

CFZ served on the Scientific Advisory Board of Sage Therapeutics and had equity in the company. Sage Therapeutics did not fund or participate in this work. PN is a co-founder of NitroTherapeutics, Inc., which aims to develop nitrous oxide as a treatment for mood disorders. Other authors have no conflicts of interest to declare.

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## Supplementary information

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[Supplemental Figure 1](#)

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