Network-Level Effects of Nitrous Oxide in the Human Brain
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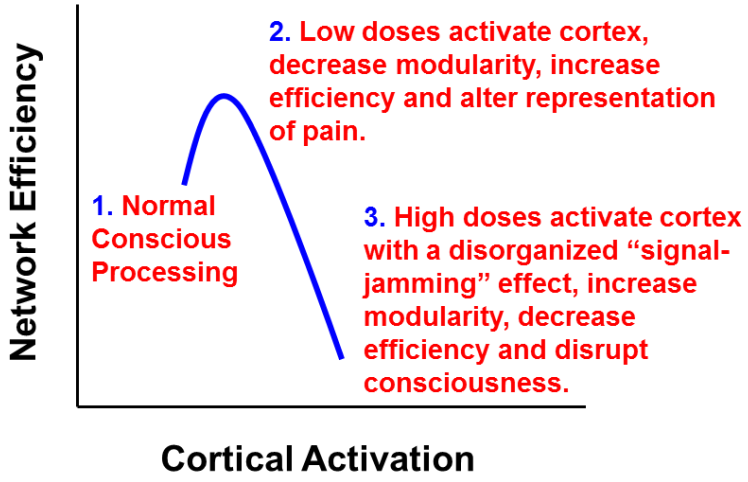
BACKGROUND

General anesthetics can, in coarse terms, be mechanistically classified as "GABAergic" and "non-GABAergic." Clearly there is great diversity among GABAergic anesthetics such as propofol and the halogenated ethers. However, in general these drugs tend to be potent hypnotics and weak analgesics that are associated with a slow-wave EEG pattern at concentrations consistent with surgical anesthesia. For nitrous oxide, the GABA receptor is not the primary therapeutic target;[1-4] this drug is a relatively weak hypnotic and potent analgesic that activates higher-frequency activity of the EEG. *Despite these differences, nitrous oxide is pharmacologically classified and clinically used as general anesthetics, alongside propofol and the halogenated ethers*. The study of anesthetic mechanisms will simply not be complete without a neuroscientific understanding of these drugs and the use of fMRI and EEG is the best technology available for systematic human studies. Identifying common neural principles of the anesthetic action of nitrous oxide and GABAergic drugs would be a major advance for the field of anesthesiology that would, for the first time, establish a common and *empirically-demonstrated* neurobiology of anesthetic-induced unconsciousness. This has not been possible with prior molecular or neurophysiological approaches, which motivates our focus on network-level properties.

Of further clinical and scientific interest, nitrous oxide disrupts pain processing at subanesthetic doses. Over the past two decades there has been increased excitement about techniques that probe the functional,[5] structural,[6] and molecular[7] mechanisms involved in human pain and its amelioration. However, less attention has been focused on how brain networks mediate pain and, moreover, how these same networks are modulated by centrally-acting analgesics such as nitrous oxide. We propose that the dose-dependent analgesic and anesthetic actions of these drugs can be understood as emergent network properties. The hypotheses motivating the currently proposed studies are summarized in the *figure on the right*.



Data derived from chronic centralized pain states suggest that pain occurs when particular brain regions functionally connect with other networks of the brain. Our recent work in fibromyalgia patients[5,8] has found that the insula, a key higher-order sensory structure, is hyperconnected to the brain's Default Mode Network (DMN), a constellation of brain regions involved in self-referential thinking (when an individual is not actively engaged with their external environment). Importantly, this enhanced connectivity is associated with self-reports of clinical pain intensity, and successful pharmacologic

treatment is associated with alternative network connectivity patterns (see preliminary data).[9] Therefore, like consciousness and anesthesia, pain and analgesia can be understood by examining brain networks and their alterations.

Relevant Literature: The relevant literature for this study pertains to the brain mechanisms for both analgesic and anesthetic doses of nitrous oxide.

There is one neuroimaging study that address the analgesic role of nitrous oxide (Gyulai et al[20]). In this study, the authors sought to identify the regions of the brain associated with the antinociceptive effects of nitrous oxide to a noxious heat stimulus. Measuring cerebral blood flow, Gyulai et al found that heat stimulus increased cerebral activation of the thalmus, anterior cingulate and supplementary motor area. Although nitrous oxide increased activation of subregions of the prefrontal cortex, nitrous oxide administered while the heat stimulus was applied reduced activation in the thalamus and supplementary motor area. As this study speaks to the analgesic effects of nitrous oxide, it does not measure the brain network properties of nitrous oxide administration at the anesthetic dose.

Preliminary Studies: We have shown previously that fibromyalgia patients display increased resting state connectivity between pain regions such as the insula and other non-pain related areas such as the DMN.[5,8] Moreover, reductions in this connectivity were associated with reductions in clinical pain: the degree of this connectivity was directly correlated with clinical pain ratings. In our *Anesthesiology* article,[9] we examined the effect of pregabalin, an efficacious analgesic, on chronic clinical pain in fibromyalgia. Prior to and following each treatment period (drug/placebo), patients underwent fcMRI of the entire brain during rest. We placed a seed voxel within the posterior insula, a region where we previously observed elevated glutamate in chronic pain patients,[7] and we assessed connectivity of this structure to the DMN before and after pregabalin and placebo. Interestingly, we found that during pregabalin treatment, but not placebo, patients displayed decreased insula connectivity to the inferior parietal lobule (IPL; a DMN region), which was paralleled by reductions in clinical pain: patients with greater reductions in insula-DMN connectivity displayed greater analgesia. *These findings are relevant to the proposed study because they demonstrate that we can measure changes in brain networks during pharmacologic treatment and these changes are related to analgesic drug action*.

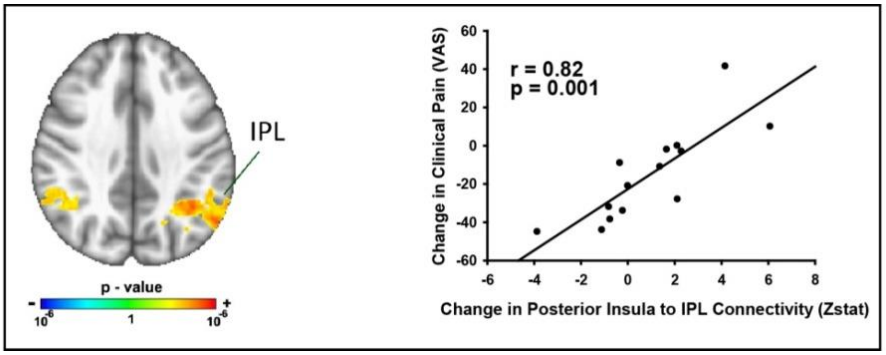


Figure above shows that change in connectivity between the posterior insula and IPL correlates with change in spontaneous clinical pain. We acknowledge that network perturbation by nitrous oxide may involve other brain regions and networks.

OBJECTIVE

The *objective of this study* is to identify the network transformations that account for the dose-dependent analgesic and sedative-hypnotic effects of the non-GABAergic anesthetic, nitrous oxide. Our *hypothesis* is that analgesic doses of nitrous oxide increase network efficiency and disrupt normal pain processing, while higher doses significantly reduce efficiency and disrupt normal conscious processing. Our *approach* is to administer two dosing levels of nitrous oxide during the acquisition of fMRI and EEG. To differentiate analgesic action of nitrous oxide from other processes, we will compare changes in brain response to noxious pressure stimuli. Our approach is *innovative* in that no study to date has acquired fMRI or EEG data to assess non-GABAergic anesthetics or to assess the relationship between analgesia and anesthesia. The *rationale* for this study is that, once completed, we will have established a detailed understanding of the therapeutic effects (analgesia and anesthesia) of a unique and understudied class of anesthetic drugs. Our *expectation* is that the knowledge derived from this study will have a positive impact by filling in a major gap in our understanding of anesthetic mechanisms and will potentially facilitate a generalized framework for anesthetic-induced unconsciousness. Furthermore, the study will provide a sophisticated network-level understanding of the relationship between the neural states of analgesia and anesthesia.

SPECIFIC AIM

1. To identify dose-dependent changes in brain networks using fMRI studies in healthy volunteers receiving nitrous oxide.
2. To identify dose dependent changes in brain feedback connectivity using EEG in healthy volunteers receiving nitrous oxide.

METHODOLOGY

Here we describe a mechanistic study in healthy human volunteers to achieve the objectives of our specific aims.

Participant Recruitment: We will recruit 25 individuals for an fMRI/EEG study.

Healthy volunteers will be identified through the UM Clinical Studies website (http://www.umclinicalstudies.org) for research volunteers. We will also utilize the Minority Health Research Program (MHRP) to help recruit minorities into our study. Our goal is to recruit groups of subjects that are representative of the southeastern Michigan population. (See Appendix A for ethnic and racial targeted enrollment). All interested volunteers will first contact Dr. Mashour or the study coordinator for a preliminary screen to determine if the subject meets the inclusion/exclusion criteria and that the subject understands the research study. Specifically, the screening script will be read to potential participants, after which, they will be screened over the phone for eligibility using phone screening questionnaires. All screening documents have been attached to the Subject Recruitment section of the IRB application. Dr. Mashour or the study coordinator will also answer any questions that the potential participants may have at this time. If the potential participant responds to the psychosis, the mood disorder, or the Patient Health Questionnaire-9 (PHQ-9) depression screening questionnaire in

a manner that would potentially indicate a depressive/mood diagnosis, he/she will be referred to the Depression Center at the University of Michigan Health System (1-800-525-5188). Eligible participants will then be scheduled to participate. Dr. Mashour and the study coordinator are responsible for obtaining full written informed consent on the day of the first study visit upon the participant's arrival. Privacy will be maintained throughout the screening process per normal UMHS HIPAA privacy policies.

Study participants:

Inclusion criteria include: age 21-40, right-handed, American Society of Anesthesiologists Class 1 physical status, body mass index <30, with Mallampati 1 or 2 airways.

Exclusion criteria include: history of obstructive sleep apnea, history of difficult airway with previous anesthetics, gastroesophageal reflux, hypertension or other cardiovascular abnormalities, pulmonary hypertension, history of drug use, history of chronic alcohol abuse, history of pain disorders, history of depression, psychosis or bipolar disorder, history of seizures or other neurologic disorders, history of methylenetetrahydrofolate reductase deficiency (a contraindication for nitrous oxide), history of known hypersensitivity to midazolam, Zofran, labetalol or glycopyrrolate, pregnant and / or nursing mothers, contraindications to the neuroimaging methods used (i.e. metal implants), tattoos in the head/neck region (all other tattoos may be subject to study exclusion by investigators), and any impairment, activity, or situation – as determined by the study coordinator or primary investigators – that would prevent satisfactory completion of the study protocol.

Power analysis: We calculated power using data from fMRI analyses. Based on an effect size (Cohen's *d*) derived from preliminary data, 22 participants are required for a 90% power to detect a statistically significant reduction in average path length (reflecting network efficiency). In case of drop out across, we will continue to recruit until we have 22 volunteers with completed data sets.

EXPERIMENTAL PROTOCOL

Each participant will participate in two study visits, one consent/pre-scan visit and one scanning visit within 3 days apart. During the consent/pre-scan visit, participants will undergo consent for participation in the study and Quantitative Sensory Testing (QST). During the scanning visit, simultaneous fMRI/EEG data will be acquired while participating in additional tasks and administration of nitrous oxide gas.

Consent/Pre-Scan Visit

The study coordinator will determine whether the volunteer meets inclusion and does not meet exclusion criteria prior to consent. Following consent, each volunteer will complete a resting state questionnaire and QST familiarization/training during EEG acquisition. MR-compatible EEG cap and electrodes will be affixed to the participant's scalp to monitor brain activity during QST training. QST will consist of a constant pressure applied to the lower leg and/or visual stimulus outside of the scanner. Following each stimulus, participants will rate the pain intensity (0 = "no pain, 100 = "worst pain imaginable") and pain unpleasantness (0 = "neutral", 100 = "extremely unpleasant"). Participants will be

asked to complete another resting state questionnaire following training on simultaneous visual stimulus and pressure cuff. During this visit, participants will also be seen briefly by an anesthesiologist for an airway assessment to check for eligible Mallampati 1 or 2 airways.

Scanning Visit

Simultaneous Functional Magnetic Resonance Imaging/Electroencephalogram (fMRI/EEG)

Each volunteer will participate in one scanning visit in which simultaneous fMRI/EEG data will be collected wherein they receive placebo (20 minutes) followed by inhaled nitrous oxide at analgesic levels (35% inhaled concentration) over 40 minutes, then a nitrous oxide dose consistent with anesthetic-induced unconsciousness (70%) for 15 minutes [See Figure 1];

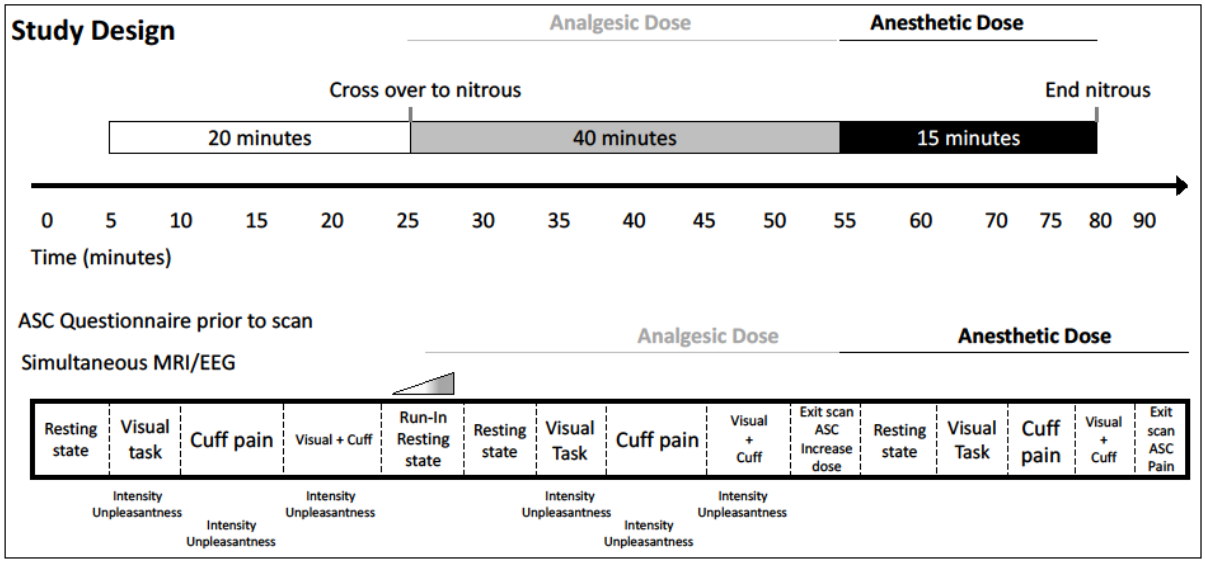


Figure 1. Study design schematic

Upon arrival, participants will complete baseline questionnaires (Part 1a). Prior to drug exposure, volunteers will be given an altered states of consciousness (ASC) questionnaire followed by a baseline resting state fMRI scan. During the placebo period, fMRI data will be acquired during additional tasks: visual stimulation task, evoked pressure pain applied to the lower leg induced by a cuff/automated tourniquet, and a combination visual stimulation and pressure pain cuff task. These scans will be repeated during both analgesic and anesthetic doses of nitrous oxide. End-tidal nitrous oxide concentrations will be assessed by standard gas analyzers during spontaneous ventilation through a secured face mask with tight seal.

All subjects will have MR-compatible EEG electrodes affixed to their scalp for monitoring during the study. Participants will first complete an altered states of consciousness questionnaire. Continuous EEG will be acquired during a baseline (rest) period followed by three additional tasks mentioned previously.

For each participant, these tasks will be repeated at a low dose of nitrous oxide that is normally associated with analgesia (35% inhaled concentration) and a higher dose of nitrous oxide that is associated with anesthesia. Following administration of the analgesic dose of nitrous oxide, participants will be asked to complete a questionnaire to assess altered states of consciousness. Each testing session will conclude with a 15-minute continuous EEG acquired during the participant's recovery of consciousness. Following testing, the participants will be asked to complete an altered state of consciousness questionnaire.

To maximize safety, nitrous oxide will be delivered using MRI-compatible anesthesia machines at the University of Michigan hospital, and anesthetic doses of nitrous oxide will be administered outside of the scanner, where airway patency and physiological stability will be established prior to imaging the state of anesthetic-induced unconsciousness. At least two fully-trained anesthesiologists will direct all anesthetic administration. In order to maximize clinical monitoring during emergence, recovery from anesthetic doses will occur outside of the MRI scanner as well. All participants will receive ondansetron (4mg IV) with an additional dose of dexamethasone (4mg IV) if needed to prevent nausea and vomiting. In addition, glycoyrrolate (0.42-0.4 mg IV), labetalol (5-10 mg/kg IV), and midazolam (1-2 mg IV) will be available for use to mitigate any side effects, as needed. Also, standard intraoperative monitors (electrocardiogram, pulse oximetry, capnography) will be used.

Subjects will only be discharged if standard clinical criteria for discharge at UMHS is met. This includes being awake, fully responsive, able to walk, able to urinate, and no significant nausea and vomiting. The medication will be given as needed during the recovery period while being monitored by Dr. Mashour or another fully trained anesthesiologist. Participants will not be allowed to drive home and transportation will be provided. In addition, approximately 24 hours after the nitrous anesthetic, the study coordinator will call the participant to follow-up and ensure that there are no adverse events. If an adverse event is detected, the study coordinator will immediately contact Dr. George Mashour and determine the appropriate course of actions to be taken.

DATA COLLECTION

Resting State Functional Connectivity (fcMRI).

During fcMRI, participants will either be at rest or receiving a pain stimulus via a pressure cuff applied to the left lower leg evoking tonic pressure with pain of 40 on a 100-point numerical rating scale (See below; 0=no pain; 100=worst pain imaginable). Functional connectivity fMRI will be performed on a 3 Tesla Philips Achieva (Best, Netherlands) using a T2* weighted echo-planar sequence with the following parameters: TR/TE = 2000/30ms, flip angle=90˚, FoV = 200x200mm, 48 AC-PC aligned slices, slice thickness 3 mm, scan time = 6min. These data will be analyzed using two approaches: a model-free independent component analysis (ICA) and seed-voxel based method (from evoked pain fMRI; See below).

Evoked pressure pain with blood pressure cuff:

Calibrated cuff pain stimuli will be delivered to the gastrocnemius area of the dominant leg using a validated cuff pain device that has been recently adapted to the MRI scanning environment. A computer controlled air compressor (Hokanson Rapid Cuff Inflator) will inflate the cuff to a pre-specified pressure, and maintain the pressure at that level. Subjects will participate in one test session of cuff pain prior to the EEG and the fMRI session. Pressure pain will be applied for a continuous 6-minute period three times during the fMRI protocol to evaluate functional brain connectivity response to deep pain (see below). One advantage to using cuff algometry pain is that unlike more superficial methods (e.g. heat pain), cuff pain responses are unaffected by sensitization or desensitization of the skin, indicating that this procedure primarily assesses sensitivity in muscle and other deep tissues. We have considerable experience applying these techniques in both healthy adults and chronic functional pain patients, and have found that all subjects are able to tolerate these procedures without any lasting discomfort.

Training Session. A training session with the cuff will be used to familiarize subjects with the stimuli and rating procedures and determine appropriate stimulus intensities to be used subsequently in the imaging session (see below). This can take place on the day of fMRI or during the in-clinic consent visit. During training, subjects will be maintained in a seated position in a chair with the left foot resting on a support at a slightly elevated position (but at a lower level than that of the hips, in order to facilitate blood circulation in the leg).

Testing will begin with an ascending series of pressure stimuli starting at 60 mmHg and increasing 20 mmHg increments. Ten seconds after the end of each stimulus, subjects will complete two 0-100 numerical rating scales: pain intensity (0 = “no pain”, 100 = “worst pain imaginable/extreme pain”) and pain unpleasantness (0 = “neutral”, 100 = “extremely unpleasant”). The ascending series will end when a pain intensity rating of >70/100 is obtained. Participants may also receive a continuous tonic pressure for 6 minutes to their lower leg using the cuff, and asked to rate the intensity and unpleasantness of the stimulus at certain intervals during the test, up to three times. A short break will be given between each pressure test.

This training session will have the effect of rendering subjects non-naïve to the experimental conditions in the imaging session, an aspect that might be argued to have some impact on the imaging results (particularly with regard to brain activity underlying cognitive and emotional functions). However, the training session has several advantages that outweigh these concerns: (1) identification and exclusion of individuals with unstable ratings, (2) a thorough training in the use of the rating scales, and (3) the reduced potential for developing experiment-related anxiety and head motion in the imaging session.

Imaging session. On the day of the imaging session, the Pain0-Pain70 pressures will be briefly recalibrated prior to scanning using procedures similar to those used during the training session.

During the fMRI scan runs, subjects will receive cuff pressures calibrated to each subject’s Pain30-50 applied for a continuous 6-min period to evaluate functional brain connectivity response to tonic deep pain. Following each cuff pain run, participants will be asked to rate the degree of pain intensity and unpleasantness on a 100-point numerical rating scale.

Visual Stimulation Task

During the visual stimulation, participations will be undergoing a 3-minute period of dynamic visual stimulation tasks which consists of alternating 20 second blocks of flashing (8 Hz frequency) blue-yellow annulus checkerboard and a static fixation cross. The checkboard will be presented at a fixed illumination level in within the range of 4.5 to 76 lux. Following stimulation, participants will be asked to rate the intensity and unpleasantness of the stimulus on a 100-point numerical rating scale.

Electroencephalogram (EEG)

All subjects will have EEG electrodes affixed to their scalp for monitoring during the study. We will use the Brain Products MR-compatible EEG system for EEG, as it offers the capability to record 64 channels of EEG data in the MR environment. This equipment has been tested and approved for safe use by the University of Michigan Biomedical Engineering Department.

The EEG electrodes and the cap to hold the electrodes will be disinfected after each use with an FDA-approved solution provided by EGI Geodesic System (there is no standard UMHS protocol for this). The EEG electrodes have minimal risk but may cause a slight irritation to the skin that will resolve on its own. Acquisition of EEG data will occur during both the consent/QST visit and the fMRI scanner visit. During the QST visit, EEG data will be acquired during rest periods and stimulus presentation. During the scanner visit, EEG will be acquired simultaneous with the fMRI data. The sampling frequency at which data will be acquired is 5000 Hz and EEG data will undergo pre-processing prior to assessment of brain network dynamics.

Questionnaire

Each participant will complete a series of questionnaires to further characterize the study participants on the day of testing prior, during and after the experiment. Before testing, participants will complete general psychometric surveys to assess personality, emotion (positive and negative affect), depression, anxiety and handedness[21-27]. All surveys have been attached to the Survey Research section of the IRB application.

During the nitrous oxide protocol, the participants will complete four additional questionnaire components; an altered states of consciousness and resting state questionnaires, as well as intensity and unpleasantness rating. The altered state consciousness (ASC) questionnaire is a survey completed by the participant on a visual analog scale (0 – 10) to assess the affective experience of nitrous oxide administration during the study. The resting state questionnaire assess resting state cognition during non-sensory experiences (resting state scan), such as mind-wandering and self-referential thinking. Intensity and unpleasantness rating will mimic the quantitative sensory testing session, given prior to each experiment, where participants are asked to rate the intensity and unpleasantness of the sensory stimuli on the Gracely Box Scale, previously used in our lab.

The ASC questionnaire should take 10 minutes to complete and will be administered at baseline, and after the analgesic and anesthetic dose periods. The resting state questionnaire will be administered

following the resting state periods and stimulus periods during the consent/QST visit. Intensity and Unpleasantness ratings will be acquired during and following both resting scans and sensory stimuli scans (cuff pain and visual stimulation).

The final survey should take less than one hour, may be completed online and should be completed within 48 hours after the study visit. This survey will contain questionnaires related to altered states of consciousness and psychometrics.

STATISTICAL ANALYSIS

Functional connectivity magnetic resonance imaging (fcMRI)

fcMRI will be performed as described in our previous publications.[5, 8, 15] During fcMRI, participants will either be at rest or receiving a pain stimulus via a pressure cuff applied to the left lower leg evoking tonic pressure with pain of 40 on a 100 point numerical rating scale (0=no pain; 100=worst pain imaginable). This sustained pain is relevant to deep musculoskeletal clinical pain and can be applied for long durations (i.e. minutes) without tissue damage.[16] Data will be corrected for physiological effects such as cardio-respiratory fluctuations that are known to influence fcMRI estimation.[17, 18] Resting connectivity network analysis will be performed using the FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) software package. All data will be corrected for motion artifacts, smoothed (FWHM 5 mm), and high-pass temporal filtered (f = 0.006Hz). *ICA Approach.* The within- and between-subject resting fMRI data analysis will be performed using ICA through Multivariate Exploratory Linear Optimized Decomposition into Independent Components (MELODIC, an FSL tool) and a previously validated dual regression approach.[19] We will apply probabilistic ICA to identify global, independent patterns of functional connectivity existent in the entire subject population. The number of independent components will be limited to 25 to reduce subcomponents. Networks of interest will be selected and the spatial independent component maps identified from the population data will be used as a spatial regressor in a general linear model of the subject's resting fcMRI data. This will allow us to estimate subject-specific spatial maps for each component. These maps will then be passed up to group analysis. *Seed-voxel Approach:* We will evaluate functional connectivity using seed-voxels from brain regions previously shown to be involved in consciousness or pain. The average time course in these regions will be used as regressors in a whole brain general linear model to find other brain regions also correlated with the seed region. A whole brain general linear model will be performed using the seed time course, averaged over a 3 mm radius sphere, as the regressor of interest. The resulting single subject maps will be normalized to standard space and passed up to group analyses. *fcMRI group analyses*: Nitrous oxide effects on brain connectivity will be estimated using a factorial model with treatment (nitrous versus placebo) and time (pre versus post) as factors. All statistical maps will be corrected for multiple comparisons on the cluster level $p < 0.05$, derived from an uncorrected $p < 0.001$ on the voxel level.

Network Analysis

In terms of network properties to be analyzed, the **degree** is the number of connections a node (in this case, a given brain region) has, while the **modularity** is a measure of the extent to which the connected regions form individual functional modules. We will first investigate the characteristic modules

corresponding to different states, then quantify the modularity of the networks. High-degree **hubs** facilitate "shortcuts" of network information transmission (much like a hub airport facilitates travel shortcuts) and are identified with the measures of *centrality* in a network. The centrality of a node measures its relative ability to integrate information globally within a brain network. We will assess several centrality measures (degree centrality, closeness centrality, betweenness centrality and Eigenvector centrality) to test the robustness of the hub structure. The **average path length**, which measures the mean number of steps along the shortest paths for all possible pairs of network nodes, will be used for quantifying the **global efficiency** of brain networks (since efficiency varies inversely with path length). Network measures will be compared across states (waking, analgesia, anesthesia) using a repeated measures two-way analysis of variance (ANOVA) and a post hoc Tukey multi-comparison test (behavioral state as the within-subjects factor, anesthetic agent as the between-subjects factor). A p value <0.05 will be considered significant.

EXPECTED OUTCOMES

Based on preliminary data, we predict that administration of *low-dose* nitrous oxide will decrease network modularity and increase network efficiency, disrupting normal pain processing. Specifically, we expect to see altered relationships of key pain structures (insula, thalamus, S1 and S2) and their connections with the DMN. We expect that, as cortical activation and disorganization increase with higher doses, nitrous oxide will increase network modularity and decrease network efficiency, disrupting conscious processing of the environment in general. *Our prior study of propofol[44] and preliminary data with 60% nitrous oxide showing decreased efficiency and increased modularity suggest the exciting possibility that GABAergic and non-GABAergic anesthetics share common network-level mechanisms to induce unconsciousness*.

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APPENDIX A

ETHNIC / RACIAL TARGETED ENROLLMENT TABLE

Study Title: Network-Level Mechanisms of Nitrous Oxide in the Human Brain

Total Planned Enrollment: 22

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Females**	**Males**	**Total**
Hispanic or Latino	1	1	2
Not Hispanic or Latino	10	10	20
Ethnic Category: Total of All Subjects *	11	11	22
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	2	2	4
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	2	2	4
White	7	7	14
Racial Categories: Total of All Subjects *	11	11	22

* The “Ethnic Category: Total of All Subjects” must be equal to the “Racial Categories: Total of All Subjects.”