Acute Intermittent Hypoxia and Breathing in Neuromuscular Disease (AIH in ALS)

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Title
Acute Intermittent Hypoxia and Breathing in Neuromuscular Disease

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Abstract
Acute intermittent hypoxia (AIH) consists of alternating periods of breathing mildly hypoxic and normoxic air, and is a well-characterized stimulus to serotonin-dependent motor plasticity (1, 2). More than two decades of research from several independent laboratories indicates AIH elicits meaningful respiratory and non-respiratory motor recovery in both animal and human models of health and neurological injury, particularly chronic incomplete spinal cord injuries (3-7). Of particular importance, the “low dose” of intermittent hypoxia represented by AIH is well below that encountered with sleep apnea, and elicits functional benefits in diverse physiological systems without detectable pathology (8, 9).

Our group is interested in the therapeutic potential of AIH for individuals with neuromuscular disease. In preliminary studies on rodent models of amyotrophic lateral sclerosis (ALS), brief AIH exposures restore lost respiratory function. Based on the efficacy of AIH in ALS rats (10, 11), and the success in translating this novel therapy to humans with chronic neurological impairment, the potential benefits of AIH in ALS patients warrant careful investigation. Most ALS patients survive less than 5 years after diagnosis, and the main cause of death is respiratory failure (12, 13). Few options are available to preserve breathing other than mechanical ventilation, which diminishes the quality of life to an extent that it is often rejected by ALS patients. Any treatment options to preserve/prolong independent breathing ability will greatly enhance the quality (and duration) of life in those suffering from ALS. Here, we seek proof of concept data that AIH may be a simple and effective therapeutic option to preserve independent breathing ability in ALS.

The fundamental hypothesis guiding this proposal is that even a single AIH trial improves respiratory (and non-respiratory) motor function in ALS patients. We propose to evaluate respiratory and limb strength, minute ventilation and respiratory muscle EMG activity in ALS patients versus healthy age and sex-matched controls in response to AIH and sham AIH (normoxia) interventions. We will investigate the ability of a single AIH trial to increase voluntary respiratory motor function (**Aim 1**). An additional, exploratory aim tests the hypothesis that ALS patients utilize distinct patterns of respiratory muscle recruitment versus healthy controls, and that these patterns are partially normalized by AIH (**Aim 2**). Both aims are designed to collect preliminary safety and feasibility data as we develop methods that improve our ability to detect and treat respiratory impairment in ALS.

Background

Acute intermittent hypoxia (AIH) consists of short, repeated presentations of reduced oxygen in the inspired gas (~9-11% oxygen, ~1 – 1.5 minutes), interspersed with normal oxygen periods (~1-1.5 min, inspired O_2=21%). AIH induced motor plasticity was first described more than two decades ago as a persistent enhancement of phrenic and hypoglossal neural activity lasting hours after AIH had ended. (14, 15) Subsequent research demonstrated that AIH preconditioning causes cumulative effects, and enhances the extent of motor facilitation induced by a single AIH presentation (16, 17). These observations triggered investigations concerning the potential of repeated AIH as a treatment to preserve and/or restore breathing ability with neural injury or disease (3, 9).

More than two decades of research on animal models have produced a detailed understanding of mechanisms giving rise to AIH-induced respiratory motor plasticity (3, 4, 6, 18, 19). Briefly, each hypoxic episode activates ventilatory chemoreflexes initiated by carotid body chemoreceptors. Carotid body sensory activity stimulates medullary respiratory neurons that increase breathing (1, 2) and raphe neurons that contain the neuromodulator, serotonin. During each hypoxic episode in the AIH protocol, this neural network is repeatedly activated. The episodic release of serotonin near respiratory motor neurons is at the heart of AIH-induced plasticity versus low oxygen *per se* (4). Episodic activation of serotonin receptors on respiratory motor neurons initiates intracellular signaling cascades that trigger new brain-derived neurotrophic factor (BDNF) protein synthesis, strengthening synapses from medullary respiratory neurons that drive breathing (1, 6). Similar mechanisms occur in both respiratory and non-respiratory motor neurons as confirmed in numerous studies in both normal (3, 20, 21) and injury/disease rodent models (3, 22). When AIH is repeated daily for many days-to-weeks, the magnitude and duration of plasticity is amplified (22).

The dose of intermittent hypoxia is critical in determining the direction and extent of plasticity, and the potential for adverse events (8). For example, high doses of intermittent hypoxia simulating obstructive sleep apnea [80 or more episodes per day/night; severe hypoxemia within each episode] elicit pathology, including systemic hypertension, neuroinflammation, and cognitive impairment (23, 24). On the other hand, respiratory motor plasticity is undermined by severe intermittent hypoxia protocols due to neuroinflammation (25). **In striking contrast**, repeated, low dose AIH [<15 episodes per day; modest hypoxemia per episode] elicits neuroplasticity without detectable pathology (26-31). To the contrary, repetitive low dose AIH exerts beneficial effects in multiple organ systems by independent research groups (for review see: (8)).

Here, we propose to study mechanisms of respiratory plasticity associated with a single presentation of mild AIH. AIH is simple to apply and well tolerated by most individuals. In eight studies on humans with chronic spinal cord injury published to date, an AIH protocol similar to that proposed here has not been associated with any adverse events (5, 7, 31-35). Several other completed, but not yet published studies from multiple laboratories increase our collective experience with (and confidence in) this protocol (Christensen et al., submitted; Sandhu et al., in preparation; Ahmed et al., in preparation). All available evidence is consistent with the conclusion that AIH protocols similar to that proposed here are safe in humans, although we do not yet have experience with ALS

patients.

Accumulating evidence demonstrates that a similar AIH protocol to that proposed here induces respiratory motor plasticity in humans. For example, in healthy sleeping adults, tidal volume and ventilation acutely increase during each hypoxic episode, and remain above baseline >20 minutes post-AIH (36). Sustained motor facilitation is also observed in genioglossus EMG activity, presumably stabilizing the upper airway (37). Using a slightly different AIH protocol (with a background of mild hypercapnia), prolonged increases in tidal volume (and ventilation) are observed in awake humans with chronic spinal injury (34). In adult patients with chronic incomplete spinal injury, a single, 30-minute AIH session (15, 1 min episodes) elicited >80% gain in ankle torque generation and EMG burst amplitude for up to 4 hours post-AIH (7). AIH-induced gains in limb muscle torque correlate with enhanced motor recruitment (5). **Here, we propose to study effects of a similar, single AIH session in normal subjects and ALS patients to gain proof of principle that AIH elicits plasticity and meaningful functional benefits in this patient population.** Although repeated, daily AIH exposure for 5 days extend the duration of motor enhancement to weeks (35), we do not yet feel justified in exposing ALS patients to such prolonged protocols pending the outcome of these initial experiments.

Based on abundant experience in humans, the AIH protocol proposed here is noninvasive, easy to administer and, importantly, has no history of adverse effects. Further, when people with chronic spinal cord injury are asked which day they had received AIH versus sham normoxia treatment, they are unable to tell (their guess has a 50/50% chance of being correct); thus, AIH at these levels is not stressful (indeed is barely detectable) for those with neurologic injury. The most notable discomfort reported during an AIH protocol is wearing the respiratory mask. Collectively, evidence from both animal models and humans with neurologic injury support the case that AIH is a safe, simple (non-invasive) and effective means of improving both respiratory and non-respiratory motor function, and that it has the potential to at least transiently improve motor function in diverse clinical disorders that compromise movement (9).

Early results (n=6) indicate respiratory motor gains in some, but not all subjects following AIH. While the primary means of AIH motor facilitation occurs via serotonergic signaling, AIH motor facilitation can occur through an alternative, adenosine-mediated signaling pathway (38, 39). The serotonergic and adenosine pathways compete for dominance via cross-inhibition, and when activated equally, they cancel one another (19, 40). However, recent basic science data from our group indicates that pre-treatment with an adenosine receptor antagonist can restore AIH-induced respiratory motor function (41). Aim 3 is an optional/exploratory aim evaluating whether co-administration of low-dose (3 mg/kg) caffeine, a non-selective adenosine receptor antagonist, augments respiratory and grip function to a greater degree than AIH alone. Caffeine is rapidly absorbed, and then degraded to paraxanthine in the liver via Cytochrome P450 1A2 (CYP1A2). Maximal serum concentrations are reached within ~1 hour of consumption.(42) The plasma half-life of caffeine is ~4 hours, but ranges from 2-8 hours between individuals.(43) Caffeine freely crosses the blood-brain barrier and enters the CNS; 3 mg/kg of caffeine inhibits A_{2A} (~60%) and A_1 receptors (50%); 6 mg/kg of caffeine yields ~75% A_{2A} and ~65% A_1 receptor inhibition.(44) Caffeine also inhibits phosphodiesterase activity, but only at doses >9 mg/kg.(45) Caffeine is the most widely

used psychoactive drug worldwide (46), and is considered safe for use in most neurodegenerative disorders, including ALS (47, 48).

Preliminary studies in rodent models of ALS:
Diminished breathing ability can be partially restored by harnessing the intrinsic capacity for plasticity (1). In rats with incomplete cervical spinal injury, even a single presentation of AIH increases phrenic/diaphragm function ipsilateral to injury (22, 49). Repetitive AIH for one week enhances both the magnitude and duration of this effect (20, 31, 35). The mechanism of functional recovery is a shifting function of time post-injury; for example, 1 week of daily AIH beginning 1 week post-injury improves breathing capacity by an adenosine dependent, serotonin-independent mechanism (31, 50). On the other hand, 1 week of daily AIH elicits functional improvement of breathing capacity by a serotonin-dependent, adenosine constrained mechanism. Thus, multiple, time-dependent mechanisms can contribute to the functional plasticity observed. Both mechanisms of functional recovery are transient, although prolonged repetitive AIH exposures appear to elicit more robust and prolonged functional recovery. We are currently exploring optimal protocols with respect to both the amplitude and duration of functional improvement.

Preliminary studies demonstrate that even a single AIH exposure restores diminished phrenic nerve activity during late-stage disease in a rat model of ALS, the $SOD1^{G93A}$ transgenic rat (10, 11, 51, 52). Indeed, in end-stage rats, AIH-induced phrenic motor facilitation is nearly double that found in wild-type littermates or in mutant rats during early stage disease (52). In end-stage ALS rats, enhanced AIH-induced phrenic motor facilitation utilizes the same serotonin-dependent mechanisms as in wild-type rats (10). These preliminary data and proof of concept studies in humans with incomplete spinal cord injury provide a strong rationale to evaluate responses to a single session of AIH in patients with ALS. Our initial investigations on a single AIH presentation will guide efforts as we work to evaluate repetitive AIH as a potential therapeutic modality in humans with ALS.

Our plan:
As a relentlessly progressive neuromuscular disease that leads to paralysis from motor neuron death, any strategy to preserve motor function for as long as possible will greatly impact the quality of life in those suffering from ALS. Current clinical ALS management includes various forms of assisted ventilation (53). However, assisted ventilation atrophies respiratory muscles, further degrading independent breathing ability (54); it takes things in the wrong direction. AIH is a potential therapy to preserve independent breathing ability, delaying the need for assisted ventilation and improving the quality and duration of life for ALS patients. We begin this line of investigation with two, limited specific aims designed to establish proof-of-principle that AIH has the intended effect.

- We will begin investigations of AIH in ALS patients by focusing on a **single AIH session** to establish proof-of-principle before considering more extensive studies of repetitive AIH, which has greater potential to alter the course of disease.
- Since the major cause of death in ALS is ventilatory failure (34), and studies of AIH-induced respiratory motor plasticity led to the principle repeated AIH as a therapeutic modality (9), our initial focus will on AIH and breathing ability in ALS patients.

- We will collect additional preliminary data on limb function (grip strength) since AIH increases strength in several motor systems, potentially benefiting patients.
- Aim 3 (optional aim) will independently evaluate the effect of pre-treatment with caffeine on respiratory and non-respiratory motor performance post-AIH.
- Our goal with this initial project is to collect preliminary data necessary to provide proof-of-concept, and to provide the foundation for extramural grant applications.

Specific Aims

Aim 1: Test the hypothesis that a single AIH trial increases maximal respiratory and non-respiratory motor function in ALS. Dependent variables will include: maximal inspiratory pressure (MIP) and maximal handgrip force (MHGF). We will address the following questions:

a. Does AIH increase voluntary inspiratory pressure generation?
b. Does AIH increase maximal handgrip force?
c. Are baseline MIP and MHGF inversely related to AIH-induced facilitation?

Aim 2: Test the hypothesis that a single AIH trial increases resting respiratory motor function in ALS. Dependent variables will include: resting tidal volume, respiratory EMG at rest, resting ventilatory drive. Three questions will be addressed:

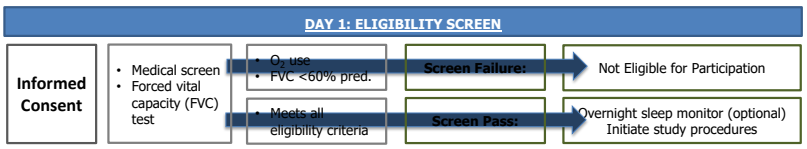
a. Does AIH increase resting tidal volume?
b. Does AIH increase the resting ventilatory drive?
c. Does AIH increase respiratory muscle activation and restore more normal respiratory muscle recruitment patterns?

Aim 3: Test the hypothesis that pre-treatment with caffeine will augment respiratory and non-respiratory motor function. Dependent variables will include: maximal respiratory pressures, maximal grip force, resting tidal volume, EMG. Three questions will be addressed:

a. Does acute consumption of caffeine enhance AIH-induced facilitation of respiratory and non-respiratory motor function?
b. Is there a difference in AIH-induced facilitation of respiratory and non-respiratory motor function between slow and fast caffeine metabolizers?
c. Does acute consumption of caffeine improve the AIH-induced increase in respiratory muscle activation?

Research Plan

Figure 1: Proposed screening procedures.



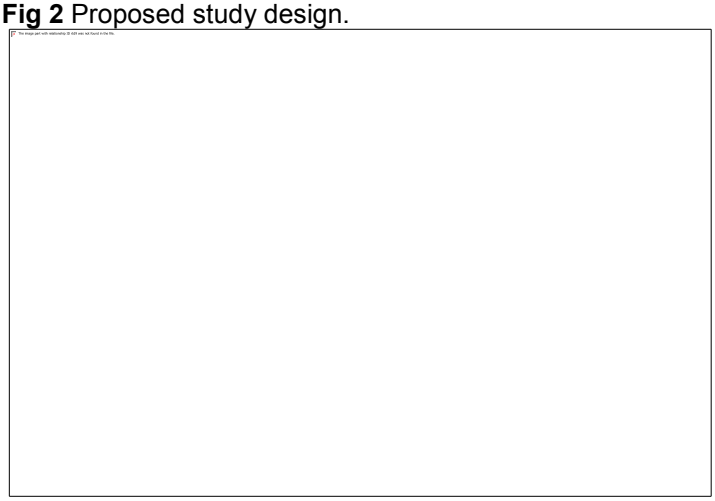
Design: Subjects will be screened for eligibility on Day 1 (**Figure 1**); those who meet the criteria will continue with the study.

The study design (**Figure 2**) will consist of up to three separate testing sessions, separated by approximately two weeks (+/- 3 days). One session will be a single, ~45-minute AIH protocol. The other will be 45 minutes of Sham AIH (ie. normoxia). Session order will be random. A third, optional session will be offered to eligible subjects (caffeine + AIH). Each session is expected to last ~3 hours, including equipment setup, patient positioning and pressure relief, rest breaks, AIH/Sham AIH, and post-intervention tests.

Subjects who elect to participate in Aim 3 will be scheduled for a third study session consisting of administration of 3 mg/kg of caffeine, followed by AIH 1 hour after caffeine consumption.

Study outcomes in Aim 1 include strength tests (respiratory and handgrip; MIP and MHGF), respiratory drive ($P_{0.1}$), tidal breathing, and the hypoxic ventilatory response (HVR). In Aim 2, respiratory muscle recruitment will be tested at normoxic rest, during hypoxia, and with maximal voluntary inspiratory pressure generation. Aim 3 outcomes include maximal respiratory pressures, MHGF, tidal breathing, and saliva tests of caffeine metabolism.

Fig 2 Proposed study design.



Subjects: We propose to study 18 ALS patients and up to 30 healthy age/sex matched control subjects. Patients who meet study criteria will be identified by Dr. Wymer (Co-I) through the UF adult neuromuscular clinic. The study PI will screen and consent patients. The PI will review the eligibility criteria, study procedures, participation risks, and the ability of subjects to withdraw from the study at any time.

Eligibility Criteria.

Subjects will be eligible to participate if they have a clinical diagnosis of ALS or are a healthy adult; are between 21 and 75 years of age; and have a baseline FVC >60% predicted for age, sex and height. Baseline FVC is routinely performed at every clinic for ALS patients. The AIH study team (PI or coordinator) will email a PFT report request to the clinic respiratory therapist, in advance of clinic. On the day of clinic, the respiratory therapist will upload the requested PFT reports directly into EPIC. After clinic, the AIH study coordinator will locate the PFT report in EPIC and save it to the electronic study binder. If the PFT report indicates the FVC is within study criteria, the subject can be contacted for an appointment. If the FVC falls below study eligibility, the patient will not be scheduled for a screening visit.

Patients will be classified into two groups: 1) uses no form of external respiratory support (IND); and 2) and uses non-invasive external respiratory support while sleeping for at least 2 nights per week to assist night time breathing or treat sleep apnea (NIV). Since 4-6 subjects in each group may fail to meet all eligibility criteria or attend the second testing session, we request to enroll up to 18 patients with ALS, with the goal of completing all study procedures in 12 patients. In addition, we request enrollment of up to 30 healthy controls to complete all study procedures in 20 (up to 20 healthy controls may be needed as a reference set for the EMG data analysis described on *page 10*). The healthy controls will be recruited via listings for this study on CT.gov and the StudyConnect website.

Patient and control participants are ineligible if they: are pregnant, have diagnosed cardiovascular disease, have a BMI >35 kg/m^2, currently take selective serotonin reuptake inhibitors (SSRI), have a history of seizures, have a history of hospitalization for sepsis, had a respiratory infection or took antibiotic medications within the past 4 weeks, use external respiratory support during any waking hours, participate in a pharmaceutical trial to treat ALS, or have any other medical condition the PI (Smith) or medical director (Wymer) identify would make it unsuitable to participate.

Additionally, subjects will be ineligible to participate in Aim 3 (caffeine+AIH) if they: report negative side effects to caffeine consumption (e.g. anxiety, dizziness); have a diagnosis of diabetes (due to effect of caffeine on glucose metabolism (55) or hepatic cirrhosis (impaired metabolism of caffeine (56), or take antiarrhythmic or bronchodilator medications (57).

The sample size is consistent with the time and budget required to acquire strong pilot data for an NIH R01 grant, which is the major goal of this project.

Eligible subjects who agree to participate and sign the informed consent form approved by UF will undergo further screening procedures prior to beginning study procedures. Screening will include a detailed medical and pharmacological history, caffeine consumption questionnaire, forced vital capacity (FVC) testing and resting 3-lead ECG. The likelihood of obstructive sleep apnea will be evaluated using the STOP-BANG questionnaire. Subjects will be informed of their STOP-BANG questionnaire results, and if sleep apnea is suspected, then they will be referred for possible clinical treatment (which is outside of the scope of this study). If they choose to pursue clinical treatment for sleep apnea, then they will be included in the study after 3 months of treatment have elapsed. If they choose not to seek treatment, they will be included in the study. This plan is consistent with recommendations of a recent Intermittent Hypoxia workshop considering the inclusion/exclusion of subjects with sleep apnea; recommendations were made in based on input from two nationally known sleep apnea experts participating in that workshop (Drs. David Gozal, University of Chicago, and S. Badr at Wayne State University).

At the screening session, subjects who meet all study criteria will be invited to participate in a third study session consisting of caffeine + AIH. Subjects who decline to participate in Aim 3 remain eligible to participate in Aims 1-2.

Screening Tests

- *Forced vital capacity* – the maximal volume of gas that can be forcefully exhaled, following full inspiration. FVC is a standard measure for clinical care and research, but lacks sensitivity in mild neuromuscular diseases, including ALS (58). Upright FVC will be conducted as per published American Thoracic Society and European Respiratory Society (ATS/ERS) guidelines (59-61). Study team will conduct FVC testing on screening/study visit #1 when any of the following conditions are present: (1) the most recent VC is <63% predicted, (2) most recent VC is >30 days from screening, or (3) written verification of the clinic VC is unavailable. Individuals with an FVC <60% of predicted will be ineligible for further participation in this study.
- *3-lead EKG* – cardiac rate and rhythm will be evaluated. The presence of resting tachycardia (HR>110 bpm), atrial fibrillation or other dysrhythmias will exclude

subjects from participation.

- *Sleep apnea screening. Subjects*
  - *STOP-BANG questionnaire* - a validated screening tool for OSA (62). All subjects must complete the STOP-BANG questionnaire, which consists of 4 yes-no questions regarding the presence of symptoms of OSA, plus 4 questions regarding known physical risk factors for obstructive sleep apnea. Individuals who present with a high risk for OSA and are currently not receiving treatment (e.g. overnight positive pressure ventilation or mouthpiece) will be referred to a clinical sleep specialist for further evaluation.
  - *Overnight sleep monitor* – subjects will have the option to wear an overnight sensor that non-invasively monitors arterial oximetry and actigraphy (WatchPAT, Itamar Medical), to estimate the severity of sleep-disordered breathing. Home-based peripheral arterial tonometry has a high specificity/sensitivity for detecting OSA (63). If clinically-significant OSA is detected by the sleep monitor (an apnea-hypopnea index >10 events/hour), subjects will be referred to a clinical sleep specialist for further evaluation.
  - Caffeine consumption questionnaire- subjects will be asked about their usual daily caffeine consumption habits. We will also ask the subjects about their possible reactions after consuming caffeine. This questionnaire will help us understand the responsiveness of the subjects to caffeine and help rule out individuals with adverse reactions to caffeine.

Independent Variable - Acute Intermittent Hypoxia (AIH) vs Sham AIH

Subjects will undergo a single session of AIH and a single session of Sham AIH (**Figure 3**). Test sessions will be scheduled two weeks apart at the same time of day. Subjects will be instructed to avoid caffeine and nicotine products for >6 hours prior to and avoid eating within 30 minutes of test sessions. Subjects will sit upright in a chair with the head and trunk supported while breathing through a sealed face mask. The mask will be connected to a pneumotachograph and pressure transducer (Hans Rudolph) to measure respiration, and a commercially-available hypoxicator (Hypoxico, Inc.) will provide either hypoxic or normoxic gas mixtures. Respiratory rate, end-tidal CO_2, heart rate, SpO_2, and respiratory muscle EMG will be continuously monitored during the testing sessions (PowerLab, ADInstruments).

Fig 3. Schematic of AIH and Sham AIH interventions. Each AIH session will consist of 1-minute intervals of hypoxia (9-12% O_2, shown in blue) separated by up to 2-minute intervals of normoxia. Sham AIH will use identical equipment, but the hypoxicator machine will deliver room air continuously.

1. *AIH.* A single, 45-minute AIH session will consist of 15, 1 minute hypoxic episodes (O_2=9-12%) separated by up to 2 minutes of normoxia (O_2 =21%) (to enable SpO2 to return to at least 92% for 1 full minute). The oxygen content of the hypoxic gas will be set at 9%, and adjusted upwards if necessary to maintain

SpO_2 above 78% for safety reasons. We do have reasons to believe that the specific SpO_2 within this range is an inverse indicator of therapeutic efficacy since the proximate stimulus to plasticity is activation of carotid body chemoreceptors (with neural activation) versus tissue hypoxia per se. If SpO_2 levels below 78% are observed, the hypoxic episode will end early and the hypoxicator will be adjusted to raise that value prior to the next episode. Healthy individuals and patients with spinal cord injury typically have difficulty in distinguishing room air from the mild hypoxic conditions proposed here.

2. *Sham AIH.* Sham AIH will consist of 45 minutes of normoxic gas delivered by the hypoxicator through the identical facemask system with identical procedures (other than the level of inspired oxygen per se).
3. *Caffeine + AIH.* Following baseline testing, subjects will consume 3 mg/kg of caffeine. Caffeine capsules will be prepared for each subject based upon the weight recorded at the screening visit. AIH will then commence in the same pattern as described above beginning 1 hour after caffeine consumption.

Aim 1 Dependent Variables – Maximal Voluntary Contractions

- *Maximal respiratory pressures* – maximal voluntary static contractions of the inspiratory or expiratory muscles against a closed valve, measured at the mouth (64). Sniff nasal inspiratory pressure measures inspiratory force generation at the nose. This more natural maneuver has lower variability and learning effects (65) and appears to decline in conjunction with the need for nocturnal ventilation in ALS (66, 67). The test will be repeated until 3 measurements are obtained within 10% variability; a minimum 20-second rest will be provided between measurements. Maximal inspiratory pressures will be measured at baseline and 60 min post-AIH.
- *Peak cough flow* - the maximal expiratory airflow measured through a facemask during a maximal-effort voluntary cough. Subjects will repeat 3-5 voluntary coughs to achieve 3 measurements within 10% variability. The peak cough flow will be measured at baseline and 30 min post- intervention.
- *Maximal voluntary grip force* – maximal static voluntary handgrip contractions will be evaluated in a seated position with the arm at the side and elbow flexed to 90 degrees. The test will be repeated until 3 measurements are obtained with <10% variability, and a minimum 15-second rest between measurements. Significant gains in isometric force have been reported with a single AIH session in patients with spinal cord injury. Grip strength will be measured at baseline, 30 and 60 minutes post-intervention.

Aim 2 Dependent Variables – Resting/automatic respiratory motor function

- *Minute ventilation* – the pneumotachograph and pressure transducer connected to the face mask will record breath-by-breath flow, volume, mouth pressure, and breathing rate. After achieving a stable tidal volume, 5 minutes of tidal breathing will be recorded. Tidal breathing will be measured at baseline, during hypoxic episodes, immediately following AIH, and at 30 and 60 minutes post-intervention.
- *Ventilatory drive.* Respiratory drive will be estimated with pressure generation against a transiently occluded airway in the first 0.1 sec of inspiration (ie. $P_{0.1}$); subjects will

be seated upright and breathing quietly through the face mask. An inspiratory valve will be manually occluded during the expiratory phase of a breath and will be maintained until the end of the subsequent inspiratory effort. Five measurements will be conducted, with 5-15 un-occluded breaths between each $P_{0.1}$ measurement. This validated test is resistant to learning or sensory bias and reflects unaltered neuromuscular effort (68, 69). $P_{0.1}$ will be measured at baseline, 30 and 60 minutes post-intervention.

- *Respiratory muscle EMG.* Surface EMGs of the respiratory muscles (up to six muscles, bilaterally: scalene, sternocleidomastoid, 2nd parasternal, 5th external intercostal, 8th external intercostal, and diaphragm) will be recorded during the test session. The root mean square (RMS) of each muscle will be averaged. Multi-muscle activation will be calculated using a validated, vector-based analysis(70) that provides a magnitude (Response Vector) and pattern (Similarity Index) of composite EMG activity. We will then compare multi-muscle activity from each ALS subject to the Prototype Response Vector calculated as the average Response Vector and Similarity Index calculated from the pooled control subjects (71).

Aim 3 Dependent Variables

- Maximal respiratory pressures – as described in Aim 1
- Maximal voluntary handgrip force – as described in Aim 1
- Tidal breathing pattern – as described in Aim 2
- Markers of caffeine metabolism and adenosine-mediated AIH. Saliva specimens will be collected at the screening visit and processed for single nucleotide polymorphisms in three specific genes. Polymorphisms in CYP1A2 (rs762551), ADORA2A (rs5751876), and BDNF (rs6265) will be tested using a commercially available TaqMan assay with PCR instrument and genotyping analysis software.
- Salivary caffeine – Saliva specimens will be collected before baseline testing and 60 minutes post-AIH, to measure changes in salivary caffeine levels. Salivary caffeine will be analyzed using a HPLC assay. The caffeine powder used by Westlab Pharmacy will be available to UF's ICBR to create a caffeine standard for assay analysis.

Statistical Analysis

The outcomes for both aims are largely quantitative and continuous. Sample distributions and summary statistics will be calculated for each outcome, and non-linear data distributions will be transformed as needed. Study outcomes for Aims 1-2 include acute changes in voluntary strength, resting ventilation, and respiratory drive. Each of these will be evaluated with 3-way repeated measures ANOVAs. Factors will be subject group (patient vs control), AIH condition (AIH vs Sham AIH), and time (pre, 0 minutes post, 30 minutes post, 60 minutes post); Tukey's post hoc test will be used to assess individual differences. A separate ANOVA will compare the independent effect of caffeine on motor function 60 minutes post-AIH. We will work with BERD to use genetic testing results to model independent effects of caffeine metabolism genotype on AIH motor facilitation, as pilot data to estimate sample size for a larger, future project. For the analysis of sEMG Response Vector and Similarity Index, baseline FVC will be used as a covariate. These will be tested with 2-way repeated measures ANOVAs (AIH only, factors: subject group

and time). Relationships between variables will be evaluated with Pearson's correlation. Statistical significance will be $p<0.05$.

Possible Discomforts and Risks

The physiological (and perceptual) effects of ***intermittent hypoxia*** in humans are well studied at the degree and duration of hypoxia as proposed here, and appear safe and well-tolerated (7, 33). Similar AIH protocols have been shown to improve ventilation (72, 73), enhance cerebral blood flow (26), and increase muscle sympathetic nerve activity. More severe hypoxia (3-6% O_2) and recurrent (>>15 episodes per day) have been associated over time with hypertension, increased systemic inflammation and neurocognitive deficits, as seen clinically in obstructive sleep apnea (8, 74, 75). On the other hand, intermittent hypoxia protocols consisting of less severe (>9% O2) and recurrent (<15 episodes/day) are associated with the opposite outcome, including reduced blood pressure, anti-inflammatory effects and neurocognitive enhancement (8, 74, 75). The hypoxia dose and duration have been carefully selected to optimize impact while minimizing known risks. We will monitor subjects continuously to detect adverse events, if any.

While this AIH protocol is well-tolerated and appears safe in most individuals, some patients may report transient light-headedness, dizziness, or reduced vision. Some patients also report an increase in respiratory sensation (mild to moderate shortness of breath), or rarely notice an increase in breathing (tidal volume) during the hypoxic periods. During and after the AIH protocol, we continuously monitor heart rate, respiratory rate, end tidal CO_2 ($ETCO_2$) and oxygen saturation (SpO_2) as well as the rating of dyspnea. The FiO_2 will be closely titrated to maintain SpO2 >78% during hypoxic episodes. While excessive HR and BP responses have not been reported, AIH will be stopped in the unlikely event the heart rate increases or decreases >30% from the resting value, and/or systolic blood pressure changes more than 30% from the resting value. Subjects will be informed that they can request to stop the study at any time. If SpO_2 falls below 75%, the AIH intervention will be terminated and supplemental O_2 provided.

Additionally, the ***mask interface*** for administering AIH and measuring breathing can feel warm, moist, and/or confining to some individuals. To offset discomfort, the mask will be removed during rest periods and between post-AIH measurements. We prefer to use a mask interface, since subjects are able to easily communicate while using a mask and it can accommodate patients with bulbar/oral weakness. However, if subjects cannot tolerate the mask, a mouthpiece and nose clip can be offered as an alternative.

Neurologic effects of low- to moderate-dose ***caffeine*** (3-6 mg/kg) include increases in alertness, mood, reaction time, and motor function (limb and possibly respiratory muscle strength, gait). (76-79). Higher doses of caffeine elicit concurrent A_1 receptor competitive inhibition, which may increase heart rate and/or blood pressure. However, both population studies and controlled studies in heart failure indicate caffeine has either a neutral or protective effect on cardiovascular risk (80, 81) as well as arrhythmias (82), in acute doses up to 500 mg (83). Caffeine may promote feelings of anxiety or fear in some individuals. Caffeine-induced anxiety has been linked to known polymorphisms of the ADORA2A gene and is associated to an aversion of chronic caffeine consumption (84). Caffeine is also a weak, non-specific phosphodiesterase inhibitor. While phosphodiesterase

inhibition occurs only at high doses (>9 mg/kg) that exceed typical human consumption(45, 85), some individuals report stomach pain or queasiness after consuming caffeine.

To minimize possible side effects of caffeine, we will exclude subjects with known sensitivity to caffeine. This study will use a standard dose of 3 mg/kg, which is considered by FDA and EFSA (European Food Safety Authority) (86) to be a safe and well-tolerated quantity in most individuals. Additionally, caffeine is generally recognized as safe (GRAS) by FDA's regulations solely in cola-type beverages up to 0.02% (200 ppm). 21 C.F.R. § 182.1180.
The caffeine capsules will be compounded by Westlab Pharmacy, a PCAB Accredited® compounding pharmacy located approximately 2 miles from the University of Florida's campus. Capsules will contain caffeine powder and filler (cellulose gum) in order to help in the breakdown and absorption of the caffeine. Westlab Pharmacy will compound individual capsules at a dose corresponding to 3 mg caffeine per kg body weight. To meet the minimum compounding requirements of the external pharmacy, we will order 3-5 capsules corresponding to several pre-determined body weights (e.g. 5, 10, 25, 50, 60, 70, 80, 90, and 100 kg). Westlab Pharmacy will then provide subjects with the minimum number of capsules needed to approximate (but not exceed) a 3 mg/kg dose. Each subject will orally ingest the caffeine capsules with water.

Respiratory strength testing procedures increase voluntary respiratory drive of subjects, which in some patients can result in a briefly elevated heart and/or respiratory rate, and decreased SpO2, feelings of shortness of breath and exertion. Some individuals find the transient exertion caused by breathing tests to be uncomfortable or anxiety-provoking (87). These effects occur infrequently and resolve upon removal of the respiratory stimulus. Nevertheless, we will monitor vital signs during the respiratory tests and be certain that patients understand they can rest as long as they like between test bouts or stop any test at any time.

The *surface EMG electrodes* are secured to subjects with a double-sided adhesive tape, which can cause skin redness or irritation in some individuals with adhesive sensitivities. Subjects will be asked about any known skin sensitivities, and if present a hypo-allergenic tape will be used.

The sensors attached during the *ECG and overnight sleep monitoring* may also be uncomfortable and cause mild skin irritation in some individuals sensitive to adhesive tape. The study team will attach the ECG stickers. A hypo-allergenic tape will be used instead for these individuals.

Patients with ALS will be recruited from the UF ALS clinic, which encompasses a large catchment area. The study risks of *travel* for patients with neuromuscular disease include fatigue, muscle and joint pain, and difficulty with pressure relief and toileting regimens. In addition, test performance may be diminished by fatigue. Up to $200 reimbursement has been budgeted for the cost of overnight lodging for patients.

For testing of *caffeine metabolism genes*, risks are extremely small but include the risk of exposing a subject's genetic information. To offset these risks, we will de-identify the

samples, and focus upon specific single nucleotide polymorphisms (SNP) in 3 genes that have essential known functions relating to caffeine metabolism (CYP1A2 and ADORA2A) and synaptic plasticity (BDNF). Caffeine metabolism genes and salivary caffeine levels will be analyzed at UF's ICBR, to minimize exposure of a subject's genetic information.

Safety oversight will occur internally through the UF IRB-01 as well as through a data safety monitoring board (DSMB). The DSMB will be convened before enrollment of the first patient, after completion of the first three patients, and at least annually thereafter. The DSMB will include three members - 1 expert in intermittent hypoxia, one in sleep/pulmonary medicine, and one in rehabilitation. At least 1 member will be external, since a wealth of external expertise in intermittent hypoxia expertise is available and this issue is of great interest to the scientific community. Notes will be taken and available for the IRB at continuing review.

Testing and enrollment of subjects will be paused and the DSMB will be convened, if any of the following events occur: (1) a clinically significant respiratory event determined by the PI to be related to study procedures, (2) a clinically significant cardiovascular event determined by the PI to be related to study procedures, (3) a serious adverse event as defined by IRB-01. Any subsequent study activities would only be resumed when cleared or modified by the IRB-01 and DSMB.

Possible Benefits
The expected outcome of this study is an increased knowledge of the effects of AIH on short-term maximal muscle recruitment/contraction and minute ventilation. No direct benefit is anticipated for the participants. Based upon this increased knowledge of respiration and AIH, we hope to evaluate the feasibility for studying longer-term benefits of AIH on breathing in patients in ALS. This could eventually lead to the investigation of future treatments for respiratory problems in motor neuron diseases such as ALS.

Conflict of Interest
None of the investigators have identified a conflict of interest.

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