APOE4, AGE & SEX REGULATE RESPIRATORY PLASTICITY ELICITED BY ACUTE
INTERMITTENT HYPERCAPNIC-HYPOXIA

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ADDITION TO KNOWLEDGE BASE

Acute intermittent hypoxia (AIH) is a novel rehabilitation strategy to induce functional recovery of respiratory and non-respiratory motor systems in people with chronic spinal cord injury and/or neurodegenerative diseases. Since most AIH trials report considerable inter-individual variability in AIH outcomes, we investigated factors that potentially undermine the response to an optimized AIH protocol, acute intermittent hypercapnic-hypoxia (AIHH), in healthy humans. We demonstrate that genetics (particularly the lipid transporter, APOE), age and sex are important biological determinants of AIHH-induced respiratory motor plasticity.
**ABSTRACT**

**Rationale:** Acute intermittent hypoxia (AIH) is a promising strategy to induce functional motor recovery following chronic spinal cord injuries and neurodegenerative diseases. Although significant results are obtained, human AIH trials report considerable inter-individual response variability. **Objectives:** Identify individual factors (e.g., genetics, age, and sex) that determine response magnitude of healthy adults to an optimized AIH protocol, acute intermittent hypercapnic-hypoxia (AIHH). **Methods:** Associations of individual factors with the magnitude of AIHH (15, 1-min O2=9.5%, CO2=5% episodes) induced changes in diaphragm motor-evoked potential amplitude (MEP) and inspiratory mouth occlusion pressures (P0.1) were evaluated in 17 healthy individuals (age=27±5 years) compared to Sham. Single nucleotide polymorphisms (SNPs) in genes linked with mechanisms of AIH induced phrenic motor plasticity (BDNF, HTR2A, TPH2, MAOA, NTRK2) and neuronal plasticity (apolipoprotein E, APOE) were tested. Variations in AIHH induced plasticity with age and sex were also analyzed. Additional experiments in humanized (h)ApoE knock-in rats were performed to test causality. **Results:** AIHH-induced changes in diaphragm MEP amplitudes were lower in individuals heterozygous for APOE4 (i.e., APOE3/4) allele versus other APOE genotypes (p=0.048). No significant differences were observed between any other SNPs investigated, notably BDNFval/met (all p>0.05). Males exhibited a greater diaphragm MEP enhancement versus females, regardless of age (p=0.004). Age was inversely related with change in P0.1 within the limited age range studied (p=0.007). In hApoE4 knock-in rats, AIHH-induced phrenic motor plasticity was significantly lower than hApoE3 controls (p<0.05). **Conclusions:** APOE4 genotype, sex and age are important biological determinants of AIHH-induced respiratory motor plasticity in healthy adults.
INTRODUCTION

Impaired breathing is a critical health concern for individuals living with lung and/or neuromuscular injury or disease. Repetitive exposures to brief episodes of low inspired O$_2$ (acute intermittent hypoxia, AIH) induces respiratory motor plasticity, which can be harnessed to improve respiratory and non-respiratory motor function [1]. However, human studies published to date exhibit considerable variability in AIH responses; ~30-40% of all participants are low responders to AIH [2]. The fundamental goal of this study was to identify genetic biomarkers and the influence of age and sex on individual AIH responses in healthy humans.

In a published companion article, we reported that intermittent exposure to concurrent hypoxia and hypercapnia (AIHH: acute intermittent hypercapnic-hypoxia; ~9.5% inspired O$_2$; ~4.5% inspired CO$_2$) elicited robust facilitation of diaphragm motor-evoked potential, MEP, reflection volitional pathways to phrenic motor neurons, and mouth occlusion pressure in 100 msec (P0.1), reflecting automatic ventilatory control, in healthy adults [3]. Combined hypoxia and hypercapnia are more effective at triggering respiratory motor plasticity in humans [4, 5], possibly because greater carotid chemoreceptor activation augments serotonergic raphe neuron activity more than hypoxia alone [6, 7], and/or direct activation of raphe neurons by hypercapnia [8], thereby enhancing cell signaling cascades that strengthen synapses onto phrenic motor neurons. Consistent with published human AIH trials [2], ~40% of participants respond minimally to AIHH (defined as <25% increase in diaphragm MEP amplitudes). Since clinical trials investigating rehabilitation interventions often fail due to response heterogeneity [9-11], identifying biomarkers associated with individual responses is essential for successful large-scale clinical trials [2].

Genomic analysis has improved healthcare precision in the treatment of cancer and other clinical disorders [12]. Similar focus on identifying genetic biomarkers to align genetic profiles or individual characteristics (age or sex) with the most effective rehabilitation strategies is lacking. Genetic factors regulate AIH-induced serotonin [13] and BDNF-dependent [14] phrenic motor plasticity in rats [15, 16], leading to the hypothesis that dysfunctional genes
affecting peripheral chemosensitivity, serotonergic function and/or BDNF/TrkB signaling 
undermine AIH-induced respiratory plasticity in humans (Figure 1). Dysfunctional genes that 
undermine neuroplasticity in other regions of the central nervous system, such as alleles coding 
for the lipid transporter apolipoprotein E (APOE), may also contribute to lower individual 
responses. For example, the APOE4 isoform is associated with Alzheimer’s disease, limited 
recovery from neural injury, impaired glutamate receptor function and limited BDNF availability 
[17].

Advancing age and sex are other characteristics that differentially affect AIH-induced 
phrenic motor plasticity in rats [18, 19]. An age-dependent sexual dimorphism could contribute 
to AIH and AIHH response variability in humans. Clear links between genetics, age and sex with 
AIH/AIHH-induced phrenic motor plasticity in rodents informs our hypothesis that human 
response heterogeneity to AIHH [3] is linked with dysfunctional single nucleotide polymorphisms 
(SNPs) in molecules known to regulate AIH-induced phrenic motor plasticity (e.g. the 
BDNFval/met mutation) as well as age and sex.

PROTOCOL AND METHODS

The present study was approved by the Institutional Review Board (IRB202000711) for human 
studies, and the Institutional Animal Care and Use Committee (IACUC202110316) for rat 
studies at the University of Florida. Human procedures were performed in accordance with the 
Declaration of Helsinki, except for registration in a database. This study is part of a larger 
research effort directed at optimizing AIH protocols with the use of AIHH in humans (see 3). For 
more information concerning methodological approaches and results, see supplementary 
material and Welch et al. [3].

Participants

Seventeen participants (age range=20-40 years, mean age=27±5 years, 9 females) signed a 
written informed consent form to participate in the study [3]. Participants with known
cardiovascular, respiratory, neurological, or infectious disease/illness, seizures, migraine (in the last 6 months), and/or metallic implants around the head, chest or shoulder region were excluded from the study. Females were screened for pregnancy. Participants were asked to refrain from caffeine consumption 8 hours prior to testing.

**Experimental Design**

A detailed description of the experimental protocol and outcome measures are described elsewhere [3] and in supplementary material. Briefly, in a single-blind, cross-over sham-controlled experiment, participants received on 2 days (separated by ≥ 3 days): AIHH (15, 1-min hypercapnic-hypoxia episodes with 1.5 min intervals breathing room air) and normocapnic-normoxia (Sham control). During AIHH, participants inspired from a Douglas bag filled with ~9.5% O₂ and 4.5% CO₂ (balance N₂). Participants breathed ambient air during Sham.

**Measures of Respiratory Neuroplasticity**

Diaphragm MEPs induced by transcranial magnetic stimulation were used to assess cortico-diaphragmatic neurotransmission [3, 20, 21]. Spontaneous respiratory drive was estimated using mouth occlusion pressure in 0.1 seconds (P_{0.1}) during resting breathing [22]. Tidal volume, breathing frequency and minute ventilation were also measured before (Pre), during and after (Post) AIHH and Sham. The magnitude of AIHH-induced plasticity was quantified as %-change from baseline [(Post-Pre)/Pre x100].

**Candidate Gene and Single-Nucleotide Polymorphism Selection**

Based on known roles of molecules in AIH-induced phrenic motor plasticity and a minimum population penetrance of 10% [3, 23, 24], we screened for 9 SNPs in genomic DNA extracted from the subject’s saliva. Seven candidate genes (Figure 1; Table 1) included autosomal SNPs in: apolipoprotein (APOE4, SNP IDs: rs429358 [T>C] and rs7412 [T>C]), prevalence: APOE4 homozygous ~11%, [17, 25, 26], APOE3/4 heterozygous ~15-25% [27, 28]; brain-derived
neurotrophic factor (BDNFval/met, SNP ID: rs6265 [C>T], prevalence ~30-50% [14, 29-31]);
neurotrophic receptor tyrosine kinase 2 (NTRK2, SNP ID: rs1212171 [C>T], prevalence ~50% [32-34]);
tryptophan hydroxylase 2 (TPH2, SNP ID: rs7305115, [A>G], prevalence 38-58% [35, 36]);
5-hydroxytryptamine receptor 2A (HTR2A, SNP ID: rs6313 [A>G], prevalence ~42% [37]);
and, paired-like homeobox 2B (PHOX2B, SNP ID: rs16853571 [A>C], prevalence ~6-14% [38]).

SNPs in sex chromosomes include male monoamine oxidase A (MAOA, SNP ID: rs5906957 [A>G], prevalence ~36% in male [39]) and female MAOA gene (SNP ID: rs1137070 [C>T], prevalence ~31% in female [40]).

DNA Extraction and Genotyping

Saliva collection and storage. Participants drool saliva was collected in a DNA/RNA shield-saliva collection kit (Genesee Inc.). Genomic (g) DNA from the saliva was extracted using a spin column-based DNA isolation kit (Zymo Quick-DNA Miniprep Kit Cat# D4069). Extracted gDNA was quantified via spectrophotometry (NanoDrop Model 2000C, Thermo Fisher Sci.) and sample purity was estimated by absorbance ratio of A260/A280 (sample range: ≥1.8-2.0). Extracted DNA was diluted to 1ng/ul concentration and used as templates in real time quantitative polymerase chain reaction (PCRs; QuantStudio3; Applied Biosystems). A 5’ to 3’ exonuclease assay in TaqMan (Applied Biosystems) was used to amplify the gene SNP of interest. SNP genotyping calls were performed with TaqMan Genotyper Software (Thermo Fisher Sci. Inc). Human DNA samples with known genotype from Coriell Institute’s Medical Research Repository were used as control identifier for TaqMan Genotyper Software.

Genotype coding used for regression analysis. Prior to applying linear model regression for SNP loci analysis, genotypes were recoded: 1) for BDNF, the “T” allele number was counted; 2) for APOE, the number of allele “C” in 2 loci, i.e. rs429358, and rs7412 were counted, and if the number was ≥3, the new variable was set to 1 (otherwise 0); 3) for NTRK2, the number of allele ‘T’; 4) for HTR2A, the number of allele ‘G’; 5) for TPH2, the number of allele ‘G’ was counted.
Since *MAOA* SNP loci (male, rs5906957 and female, rs1137070) have different localizations on the X chromosome, we stratified results based on sex and analyzed them separately. Data from *PHOX2B* SNP (rs16853571) was omitted in the analysis due to lack of gene variation in our study sample. For SNP locus analysis, variables age and sex were considered as covariates.

**Humanized ApoE Knock-in Rat Experiments**

Based on the observed association between *APOE*3/4 and impaired AIHH-induced diaphragm plasticity in humans, we performed follow up experiments in adult male Sprague-Dawley rats (345-385g; Envigo, IN, USA) with homozygous knock-in humanized ApoE3 (*hApoE3*; ID #395, n=4) or ApoE4 (*hApoE4*; ID #359, n=3). Neurophysiology experiments were performed in urethane anesthetized, paralyzed and ventilated rats at times consistent with human AIHH treatments (i.e., active phase; 12 a.m. in rats [41]). The primary outcome measure was the amplitude of integrated phrenic nerve bursts (1-min averages), taken before, during, and 30, 60 and 90 min after exposure to an AIHH protocol comparable to that delivered to humans (15, 1 min episodes of hypercapnic-hypoxia; 1.5 min intervals). Experimental details of these neurophysiology experiments are provided in the supplemental section and elsewhere [42-44].

**Statistics**

The quality of SNP genotype data was analyzed for deviations from Hardy Weinberg equilibrium using both the Exact Test and Chi-Squared Test. A single-locus analysis was used to assess the association of each SNP with treatment outcome [45]. After adjusting for age and sex, the association between %-change from baseline and SNPs was explored using a linear regression model in R software [46]. A detailed description of SNP genotype coding used for liner regression analysis is provided in the supplementary section. The association of age and sex with primary dependent variables (diaphragm MEPs and P0.1) were analyzed using a liner regression model.
Peak phrenic nerve burst amplitude was averaged over 1 min immediately before blood samples were taken at baseline and at 30, 60 and 90 min post-AIHH. Phrenic nerve burst amplitude was analyzed using absolute values and normalized as a percent change from baseline. Phrenic responses were analyzed using a two-way repeated measures ANOVA with Tukey’s post-hoc analysis (SigmaPlot, v12.0; Systat Software, San Jose, CA). Differences were considered significant when p<0.05. Data are expressed as mean ± SD.

RESULTS

Demographics, genotype and pre to post %-change in primary dependent variables (MEP and P0.1) following AIHH and Sham for each participant are presented in Table 1. A detailed report of the cardiorespiratory responses during AIHH exposure in the same set of individuals is presented in a companion paper [3]. Only genetics, age, and sex effects on diaphragm MEP amplitudes and P0.1 are presented here; age and sex effects are presented in supplementary material.

Gene SNPs Associated with Dysfunctional AIHH-Induced Plasticity

No departure from Hardy-Weinberg equilibria was observed within the screened autosome or sex chromosome loci. For brevity, and due to their associations with AIHH-induced plasticity, we report results in this manuscript for BDNFval/met, APOE4 and TPH2 SNPs. A complete summary of all SNPs and multiple regression analyses for %-change in diaphragm MEP amplitudes and P0.1 are provided in Tables 2A and 2B. One participant (participant ID: S06; Table 1) with TPH2 homozygous major “A” allele was identified statistically (Cook’s D >4) as the most influential data point in the regression for %-change in diaphragm MEP amplitudes (Figure 2). Therefore, data from S06 was not included in any analysis except for TPH2 group analysis.

BDNFval/met (rs6265). Eight participants were heterozygous, and none were homozygous for the BDNFval/met allele. No significant difference was observed between BDNFval/met
heterozygotes and individuals without BDNFval/met for %-change in diaphragm MEP amplitudes (Figure 2A; Table 2, p=0.290, t=1.090) or P0.1 (Figure 2B; Table 3, p=0.885, t=0.150).

**APOE (rs429358 and rs7412).** Five participants were heterozygous for APOE4 (i.e., APOE3/4); none were homozygous for APOE4. The APOE3/4 genotype was associated with diminished %-change in diaphragm MEP amplitudes following AIHH (Figure 2C, Table 2, p=0.048, t=-2.187). The %-change in diaphragm MEP amplitudes was 38% lower in individuals with APOE3/4 (APOE3/4+) versus individuals carrying other allelic APOE isoforms (e.g., APOE3/4). In contrast, no significant association between APOE3/4+ and %-change in P0.1 was observed (Figure 2D; Table 3, p=0.159, t=1.490).

**TPH2 (rs7305115).** Two participants were homozygous for the TPH2 major “A” allele (participant ID: S01 and S06), 8 participants were heterozygous and 7 homozygous for the dysfunctional minor “G” allele. Although not statistically significant, there was a marginal association between the presence of at least 1 “G” allele and %-change in diaphragm MEP amplitudes (p=0.063, t=-2.030). The coefficient of the TPH2 gene was -0.251, meaning responses were 25.1% lower than average with 1 “G” allele. This effect was primarily influenced by the outlier participant (S06) who was homozygous for “A” allele. No association was observed between TPH2 locus variants and P0.1 (p=0.990, t=0.002).

**Age-Sex Dimorphism in Diaphragm MEPs**

No significant relationship was found between age and %-change in diaphragm MEP amplitude following AIHH (Figure 4A; r=0.08, 95% CI= -2.47 to 3.32, p=0.758). No significant differences in diaphragm MEP amplitude change were observed with age in males (Figure 4B; r=0.24, 95% CI= -1.18 to -0.4.24, p=0.217) or females (Figure 4B; r=-0.01, 95% CI= -5.75 to 4.38, p=0.752).
However, males had significantly higher % change in diaphragm MEP amplitudes versus females, regardless of age (mean difference = 37±10.8%, F=12.17, p=0.004).

**Age-Sex Dimorphism in P0.1**

A negative correlation was observed between % change in P0.1 and participant’s age, despite the limited age range included in this study (Figure 4C; r=-0.64, 95% CI=-0.85 to -0.23, p=0.007). Each year of increasing age corresponded to a 3.9% decrease in P0.1 response. The decline in P0.1 with age was explained by male (Figure 4D; r=-0.73, 95% CI= -0.95 to -0.07, p=0.036) versus female responses (Figure 4C; r=-0.29, 95% CI= -0.83 to -0.52, p=0.480) to AIHH. Regression slope (F=1.77, p=0.210) and intercept (F=1.5, p=0.240) for % change in P0.1 were not significantly different between males and females.

**Humanized ApoE Knock-In Rats and AIHH Induced Phrenic Long-Term Facilitation**

Figure 3A shows average phrenic nerve burst amplitudes during and following AIHH. Baseline phrenic nerve amplitudes were not different between groups (hApoE3: 0.023±0.007 V; hApoE4: 0.022±0.013 V). On the other hand, AIHH elicited significant phrenic long-term facilitation in hApoE3 (p=0.025 vs. baseline), but not in hApoE4 rats (p=0.995). A significant interaction between genotype and time post-AIHH was observed in phrenic long-term facilitation magnitude (Figure 3B; F=5.93, p=0.007). AIHH-induced phrenic long-term facilitation in hApoE3 rats was significantly greater than hApoE4 at 30 min (p=0.004), 60 min (p=0.002) and 90 min (p<0.001) post-AIHH. Arterial CO2 partial pressures at baseline (hApoE3: 43.9 ± 1.5 mmHg; hApoE4: 45.7±1.2 mmHg) and 90 min post-AIHH (hApoE3: 44.4±1.6 mmHg; hApoE4: 46.2±0.4 mmHg) were not different.

**DISCUSSION**

We investigated the role of genetics, age and sex on AIHH-induced respiratory motor plasticity of both cortical (presumably volitional) diaphragm MEPs and brainstem automatic (P0.1) neural...
pathways in healthy adults. We report increased diaphragm MEP amplitudes following AIHH are
diminished in people heterozygous for the APOE4 allele and unaffected in BDNFval/met heterozygotes. Regardless of age, the % change in diaphragm MEP amplitudes following AIHH is greater in males versus females, whereas sex does not influence the magnitude of change in
P0.1. Finally, despite the limited age range in this study (20-40 years), there was a negative
correlation between age and P0.1 facilitation. Neurophysiological experiments in hApoE3 and
hApoE4 knock-in rats confirmed a causal relationship between hApoE4 genotype and impaired
phrenic motor plasticity.

**SNPs and AIH/AIHH Induced Plasticity**
To investigate SNPs that influence AIH/AIHH-induced respiratory motor plasticity, a panel of
genes was assessed chosen based on their known links to phrenic motor plasticity in rodents,
including SNPs linked to serotonin synthesis (TPH2), clearance (MAOA), or receptors (HTR2A),
a key neurotrophic factor (BDNF), and its high affinity receptor (NTRK2), as well as
chemoreceptor function (PHOX2B). A seventh gene, APOE4 was added to the panel due to its
association with impaired neuroplasticity [17], including AIH-induced phrenic long-term
facilitation [47].

No association was found between 6 gene SNPs and AIHH-induced respiratory motor
plasticity in the humans studied here. Tryptophan hydroxylase-2 (TPH2) is the rate limiting
enzyme for serotonin synthesis [36]; presence of a “G” allele in exon 7 of the TPH2 gene is
associated with reduced serotonin bioavailability [35, 48]. An apparent (but. not significant;
p=0.063) ~25% diminished response in the presence of 1 TPH2 “G” allele requires further study.

Since BDNF is both necessary and sufficient for AIH-induced phrenic motor plasticity in
rats [14], we hypothesized that the dysfunctional BDNFval/met allele undermines plasticity.
BDNFval/met is a common missense single nucleotide C>T polymorphic mutation at codon 66
of BDNF gene, resulting in amino acid methionine (Met) substituting valine (Val).

BDNFval66met or BDNFval/met mutation, impairs the pro-domain region of BDNF protein,
disrupting the normal trafficking of mature BDNF from neuron soma to dendrites [49-51]. This dysfunctional BDNF SNP is associated with reduced exercise-induced plasticity and functional recovery in people with spinal cord injury or traumatic brain injury [31, 52, 53]. However, contrary to our hypothesis, no association between BDNFval/met mutation and AIHH-induced respiratory motor plasticity was found (Figure 2A). We speculate that in healthy adults, one fully functional allele is sufficient to meet physiological demands and/or enable adequate responses to certain physiological stimuli, such as AIHH. Since no participants had homozygous BDNFval/met mutation, we cannot rule out an association between homozygous BDNFval/met and respiratory motor plasticity.

APOE is a triglyceride rich low-density lipoprotein that facilitates lipid transport between cells. APOE is highly expressed in the central nervous system, with 3 common human isoforms (E2, E3 and E4) [54]. With respect to neuroplasticity, the T to C nucleotide substitutions at APOE loci (APOE4) leads to arginine substitutions in the 112 and 159 positions (SNPs rs429358 and rs7412), and is the most consequential SNP mutation for neuroplasticity. Homozygous APOE4 allele is present in 11-14% of people, whereas heterozygous APOE3/4 allele is found in about 15-25% of people [27, 28]; In this group of study subjects, we observed a slightly higher percentage of APOE3/4 heterozygotes (~29%), which may be attributed to our small sample size. Individuals with the APOE4 allele experience diminished motor recovery following spinal cord injury versus other APOE alleles [25]. APOE4 protein isoform has been hypothesized to impair AIH-induced plasticity [47] as it reduces NMDA and AMPA receptor recycling in the post-synaptic membrane, and limits BDNF availability. A recent study in transgenic mice with knock-in hApoE4 suggested that APOE4 protein isoform is associated with impaired AIH-induced respiratory motor plasticity [47], consistent with our observation that at least 1 dysfunctional APOE4 allele was associated with 38% reduction in AIHH-induced diaphragm MEP facilitation. Thus, stratifying participants based on Mendelian randomization of known genetic risk factors may be critical for success of large phase II and III clinical trials investigating the efficacy of AIH/AIHH [56].
Causal link between APOE4 on AIHH-induced respiratory motor plasticity

To demonstrate a causal link between APOE4 and AIHH-induced respiratory motor plasticity, we performed neurophysiology experiments in hApoE4 and hApoE3 knock-in rats using a nearly identical AIHH protocol to humans (15, 1-minute episodes of hypercapnic-hypoxia during the night, or the active phase for rats). Whereas rats with hApoE3 manifested robust AIHH-induced phrenic long-term facilitation (~60% increase at 90 min post AIHH), hApoE4 rats failed to express significant plasticity. Thus, APOE4 undermines AIHH-induced respiratory motor plasticity in rats. Our data support an earlier report by Strattan and colleagues [47] where hApoE4 mice failed to express AIH-induced respiratory plasticity, despite study differences such as species (mice versus rats), plasticity-inducing protocol (AIH versus AIHH) and time of day (rest vs active phase).

Although the mechanistic link between a dysfunctional APOE4 allele and reduced spinal plasticity is not yet known, we suggest a few plausible hypotheses. APOE4 protein isoform converts microglia to a pro-inflammatory phenotype [26], which may undermine phrenic motor plasticity [55]. Further, the observation that hApoE4 mice exhibit more extensive perineuronal nets after spinal cord injury [47] suggests an alternate mechanism, and suggests a distinct therapeutic target to mitigate the dysfunctional effects of APOE4 genotype. Future studies investigating APOE4 induced pathophysiology may reveal additional targets to unlock AIHH induced neuroplasticity in APOE4 carriers.

Unlike the association of APOE3/4 and TPH2 SNPs with reduced diaphragm MEP responses following AIHH, no similar association was found between these genotypes and P0.1. This difference could be due to distinctions in the neuronal pathways utilized with transcranial magnetic stimulation (reflecting volitional control of breathing) versus automatic (bulbospinal) pathways to phrenic motor neurons and/or the correlation between participant age and P0.1 facilitation (see below), which likely obscured the influence of genetic factors.
Age-Sex Dimorphism in AIHH Induced Plasticity

Decades of rodent work demonstrate a link between age, sex and AIH-induced phrenic motor plasticity [18, 19, 56, 57]. Although our results are generally consistent with prior observations in rats, there were some interesting differences.

Diaphragm MEP responses. We observed that in healthy adults, regardless of age, corticospinal plasticity (i.e., diaphragm MEPs) was significantly greater in males versus females (mean difference=37±10.8%). Sex differences in the neural control of breathing have been observed during ventilatory challenges [58, 59] and the capacity for respiratory neuroplasticity [60, 61]. These sex differences could be caused by ovarian hormones that affect neurotransmission. In rats, hippocampal long-term potentiation is induced more readily in males versus females due to excitatory effects of testosterone [62, 63]. In females with normal menstruation, circulating progesterone reduces cortical excitability [64, 65]. During the luteal phase of menstrual cycle (high progesterone), increased inhibition and decreased facilitation of TMS responses are observed, which is indicative of increased GABAergic effects from progesterone metabolites [65]. In contrast, there is increased cortical facilitatory activity during the mid-follicular phase of the menstrual cycle (low progesterone, high estrogen). Thus, our results are in line with previous literature.

P0.1 responses. A significant decrease in AIHH-induced P0.1 plasticity was observed with increasing age; each year of age in the range studied (20-40 years) led to a fall in P0.1 plasticity of ~3.9%. This age-related drop was more pronounced in males than females. Negative pressure generation in 0.1 seconds of an occluded inspiration reflects respiratory neuromechanical drive prior to influences from breath-related sensory feedback, such as from lung or chest wall receptors [22]. Explanations for diminished AIH/AIHH-induced neuroplasticity with age observed in the present study include: 1) decreasing sex hormone
(testosterone/estrogen) levels; 2) diminished serotonergic function and/or 2) increased extracellular CNS adenosine levels.

Since changes in P0.1 reflect automatic control of breathing, it may be more equivalent to rodent phrenic long-term facilitation versus MEPs. In rats, phrenic long-term facilitation decreases as males reach middle-age, but increases in middle-aged females (when normalized for stage of the estrus cycle). Estrogen suppresses pro-inflammatory microglial activities and even mild inflammation impairs phrenic long-term facilitation. Testosterone is necessary for phrenic long-term facilitation in males because it is a substrate for aromatase-dependent CNS estrogen formation. In male rats, testosterone peaks at ~2-6 months of age, equivalent to ~18-40 years in humans, which is then followed by a gradual decline, similar to human males in the ~40-60 year age range. Since the age of our participants ranged from 20-40 years, reduced serum sex hormone levels are unlikely to explain variance in P0.1 responses; furthermore, the % change in P0.1 was not significantly different between sexes in this study. Adenosine is another major regulator of AIH-induced phrenic motor plasticity in rats. Extracellular adenosine levels in the central nervous system increase with age — greater adenosine-dependent inhibition of phrenic motor plasticity may occur, potentially explaining reduced P0.1 plasticity with age in our study.

LIMITATIONS

Rather than more common “omics” approaches, we selected a panel of 7 genes and 9 SNPs to investigate as a potential biomarker based on known roles of the relevant molecules in AIH-induced respiratory motor plasticity, and the relative penetrance of the SNPs in humans. Although this list does not include all SNPs that could affect AIH/AIHH-induced plasticity, we verify that genetic factors regulate AIHH-induced plasticity in humans, particularly APOE4. This is the first study to link APOE4 with spinal, respiratory motor plasticity in humans. Due to the number of potential SNPs that could be investigated, adequate correction for multiple...
comparisons will remain a challenge. Further, it is important to increase the age range studied beyond 40 years and to extend investigations to people living with disease or injury.

CONCLUSIONS

We provide evidence that the APOE4 allele, age and sex are important biological determinants of AIHH-induced respiratory motor plasticity in humans. The presence of one dysfunctional APOE4 allele undermines cortico-spinal respiratory motor plasticity. Experiments using humanized APOE4 knock-in rats support a causal relationship between APOE4 and impaired AIHH-induced respiratory motor plasticity. Contrary to our original hypothesis, no evidence was found for diminished plasticity in individuals with BDNFval/met mutations, although no homozygous subjects were included in this analysis. Regardless of age, males exhibited greater AIHH-induced cortico-spinal plasticity versus females; conversely, AIHH-induced plasticity in P0.1 is negatively associated with increasing age – an effect that is more pronounced in males than females. Thus, age, sex and genetic factors should all be considered when attempting to differentiate responders from non-responders in clinical trials investigating therapeutic use of AIH/AIHH in individuals with spinal cord injury or other neurological conditions. With such information in hand, it may be possible to refine rehabilitation protocols and/or provide individualized treatment strategies.
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LEGENDS

Figure 1: Conceptual diagram depicting cell signaling mechanisms (and candidate biomarker genes) for acute intermittent hypercapnic-hypoxia (AIHH) induced respiratory motor plasticity. The panel of SNPs with a population prevalence of >10% were tested for association with reduced AIHH-induced plasticity in humans. These include 6 SNPs in genes involved in AIH cell signaling: (1) raphe chemosensitive cells (PHOX2B), (2) serotonin precursors in the central nervous system (tryptophan hydroxylase-2, TPH-2), (3) serotonin clearance enzyme (monoamine oxidase A, MAOA), (4) serotonin-2A receptors (HTR2A), (5) brain-derived neurotrophic factor (BDNF) and (6) TrkB receptors (NTRK2). A seventh dysfunctional SNP in neuroplasticity related gene, APOE (APOE4), was also tested for association.

Figure 2: Relative (%-change from baseline) changes in diaphragm motor-evoked potential (MEP) amplitudes and mouth occlusion pressure (P0.1) in individuals with BDNFval/met (panels A and B) and APOE3/4 (panels C and D) SNP. No associations were observed between individuals with BDNFval/met and the change in MEP amplitudes (panel A) or P0.1 (panel B). Individuals with dysfunctional APOE3/4 allele were associated with a significantly lower AIHH-induced change in MEP amplitude (t=-2.28, p=0.048, panel C). However, no association between APOE3/4 and AIHH-induced P0.1 responses were observed (panel D). Δ=change. *p<0.05. Results expressed as mean ± SD. ♦ participant (S6) was identified as the most influential point (Cook’s D >4) in the %-change in diaphragm MEP amplitudes, therefore, the data was not included in group analyses.

Figure 3: AIHH elicits phrenic long-term facilitation in hApoE3 but not hApoE4 knock-in rats. Panel A shows average traces of phrenic nerve amplitude for hApoE3 (n=4; gray) and hApoE4 (n=3; black) knock-in rats, *p<0.050 vs baseline. Panel B phrenic burst amplitude (%-
change from baseline) in hApoE3 (gray circles) and hApoE4 (black circles) rats, \( +p<0.005 \) versus hApoE4. \( \Delta = \text{change} \). Results expressed as mean \( \pm \) SD.

Figure 4: Relationship between age and sex on the magnitude (%-change from baseline) of change in diaphragm motor-evoked potential amplitudes (MEP, panels A and B), and mouth occlusion pressure in 0.1 seconds (P0.1, panels C and D) following AIHH. No association between age and the magnitude of change in diaphragm MEP amplitudes was observed (panel A). Regardless of age, males (black line, panel B) had significantly greater responses in MEP amplitudes versus females (gray line, panel B). The magnitude of change in P0.1 reduced significantly with age (panel C); however, the decline was more pronounced in males (\( r=-0.73, p=0.036 \), black line, panel D) versus females (\( r=-0.29, p=0.480 \), gray line, panel D). \( \Delta = \text{change} \). \( *p<0.05 \). Results expressed as mean \( \pm \) SD.

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Table 1. Demographics and SNP genotype classification details. Includes individual participants’ %-change from baseline in diaphragm motor-evoked potential amplitudes (MEP) and mouth occlusion pressure (P0.1) following AIHH and Sham exposures. Genotype letters in bold and underlined text indicate dysfunctional allele.

Table 2. Association of SNPs with %-change from baseline in diaphragm motor-evoked potential amplitudes (MEP). \( *p<0.05 \).

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Figure 1: Conceptual diagram depicting cell signaling mechanisms (and candidate biomarker genes) for acute intermittent hypercapnic-hypoxia (AIHH) induced respiratory motor plasticity. The panel of SNPs with a population prevalence of >10% were tested for association with reduced AIHH-induced plasticity in humans. These include 6 SNPs in genes involved in AIH cell signaling: (1) raphe chemosensitive cells (*PHOX2B*), (2) serotonin precursors in the central nervous system (tryptophan hydroxylase-2, *TPH-2*), (3) serotonin clearance enzyme (monoamine oxidase A, *MAOA*), (4) serotonin-2A receptors (*HTR2A*), (5) brain-derived neurotrophic factor (*BDNF*) and (6) TrkB receptors (*NTRK2*). A seventh dysfunctional SNP in neuroplasticity related gene, APOE (*APOE4*), was also tested for association.
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Table 1. Demographics and SNP genotype classification details. Includes individual participants’ %-change from baseline in diaphragm motor-evoked potential amplitudes (MEP) and mouth occlusion pressure (P_0.1) following AIHH and Sham exposures.

Genotype letters in bold and underlined text indicate dysfunctional allele.

| ID  | Age | Sex | BDNF rs6265 (Alt. Allele=T) | APOE rs429358 (Alt. Allele=C) | APOE rs7412 (Alt. Allele=C) | APOE classification (rs429358+rs7412) | NTRK2 rs1212171 (Alt. Allele=T) | HTR2A rs6313 (Alt. Allele=G) | PHOX2B rs16853571 (Alt. Allele=C) | TPH2 rs7305115 (Alt. Allele=G) | MAOA Male rs5906957 (Alt. Allele=A) | MAOA Female rs1137070 (Alt. Allele=T) | %-change from baseline in Diaphragm MEP | %-change from baseline in P_0.1 |
|-----|-----|-----|-----------------------------|-------------------------------|-----------------------------|-----------------------------------|---------------------------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| S01 | 40  | M   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | GG                            | AA                              | AA                              | GG                              | AA                              | 150.3                          | 130.8                         |
| S02 | 29  | F   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 100.9                          | 107.9                         |
| S03 | 28  | F   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 117.3                          | 107.7                         |
| S04 | 27  | F   | CC                          | TT                            | CT                          | APO-E2/E3                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 215.5                          | 195.9                         |
| S05 | 30  | F   | CC                          | TT                            | CT                          | APO-E2/E3                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 156.0                          | 147.0                         |
| S06 | 24  | F   | CC                          | TT                            | CT                          | APO-E2/E3                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 142.7                          | 125.5                         |
| S07 | 30  | F   | CC                          | TT                            | CT                          | APO-E3/E4                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 69.3                           | 52.6                          |
| S08 | 21  | M   | CC                          | TT                            | CC                          | APO-E3/E4                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 85.3                           | 127.0                         |
| S09 | 36  | F   | CC                          | TT                            | CT                          | APO-E3/E4                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 91.4                           | 63.6                          |
| S10 | 24  | F   | CC                          | TT                            | CT                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 137.7                          | 103.8                         |
| S11 | 32  | F   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 110.7                          | 93.4                          |
| S12 | 34  | M   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 106.4                          | 89.9                          |
| S13 | 21  | M   | CC                          | TT                            | CT                          | APO-E3/E4                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 94.2                           | 70.3                          |
| S14 | 24  | M   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 120.2                          | 82.4                          |
| S15 | 23  | F   | CC                          | TT                            | CC                          | APO-E3/E3                         | CT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 164.4                          | 87.5                          |
| S16 | 22  | M   | CC                          | TT                            | CC                          | APO-E3/E4                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 141.4                          | 73.4                          |
| S17 | 26  | M   | CC                          | TT                            | CT                          | APO-E3/E3                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 91.1                           | 104.9                         |
| S18 | 31  | F   | CC                          | TT                            | CT                          | APO-E2/E3                         | TT                              | AG                            | AA                              | AA                              | GG                              | CT                              | 112.6                          | 94.7                          |

** Outlier
### Table 2. Association of SNPs with % change from baseline in diaphragm motor-evoked potential amplitudes (MEP). *p<0.05.

<table>
<thead>
<tr>
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<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>P value</th>
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### Table 3. Association of SNPs with % change from baseline in mouth occlusion pressure in 0.1 seconds (P0.1). *p<0.05.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
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REFERENCES


