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APOE4, Age, and Sex Regulate Respiratory Plasticity Elicited by Acute Intermittent Hypercapnic-Hypoxia

Jayakrishnan Nair[1,2], Joseph F. Welch[1,3], Alexandria B. Marciante[1], Tingting Hou[4], Qing Lu[4], Emily J. Fox[1,5,†], Gordon S. Mitchell[1,*,†]

[1]Breathing Research and Therapeutics Center, Department of Physical Therapy, University of Florida, Gainesville, 32603, USA, [2]Department of Physical Therapy, Thomas Jefferson University, Philadelphia, PA, 19107 , USA, [3]School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Edgbaston, Birmingham, 3- B15 2TT, UK, [4]Department of Biostatistics, University of Florida, Gainesville, 32603, USA and [5]Brooks Rehabilitation, Jacksonville, FL, 32216, USA

[*]Address correspondence to G.S.M. (e-mail: gsmitche@phhp.ufl.edu)

[†]Co-senior authors.

Abstract

Rationale:

Acute intermittent hypoxia (AIH) shows promise for enhancing motor recovery in chronic spinal cord injuries and neurodegenerative diseases. However, human trials of AIH have reported significant variability in individual responses.

Objectives:

Identify individual factors (eg, genetics, age, and sex) that determine response magnitude of healthy adults to an optimized AIH protocol, acute intermittent hypercapnic-hypoxia (AIHH).

Methods:

In 17 healthy individuals (age = 27 ± 5 yr), associations between individual factors and changes in the magnitude of AIHH (15, 1-min O2 = 9.5%, CO2 = 5% episodes) induced changes in diaphragm motor-evoked potential (MEP) amplitude and inspiratory mouth occlusion pressures (P0.1) were evaluated. 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*) compared to individuals with other *APOE* genotypes ($P = 0.048$) and the other tested SNPs. Males exhibited a greater

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diaphragm MEP enhancement versus females, regardless of age ($P = 0.004$). Additionally, age was inversely related with change in P0.1 ($P = 0.007$). In h*ApoE4* knock-in rats, AIHH-induced phrenic motor plasticity was significantly lower than h*ApoE3* controls ($P < 0.05$).

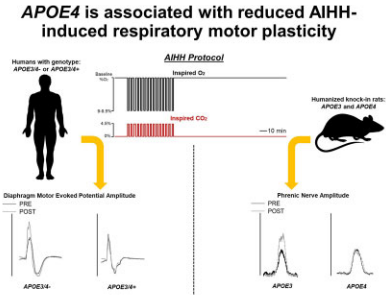
Conclusions:

APOE4 genotype, sex, and age are important biological determinants of AIHH-induced respiratory motor plasticity in healthy adults.

Addition to Knowledge Base:

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 disease. Figure 5 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, 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

Graphical Abstract



Key words: intermittent hypercapnic-hypoxia; respiratory neuroplasticity; biomarkers; genetics; *APOE4*; age; sex

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 ms (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 (eg, the *BDNF-val/met* mutation) as well as age and sex.

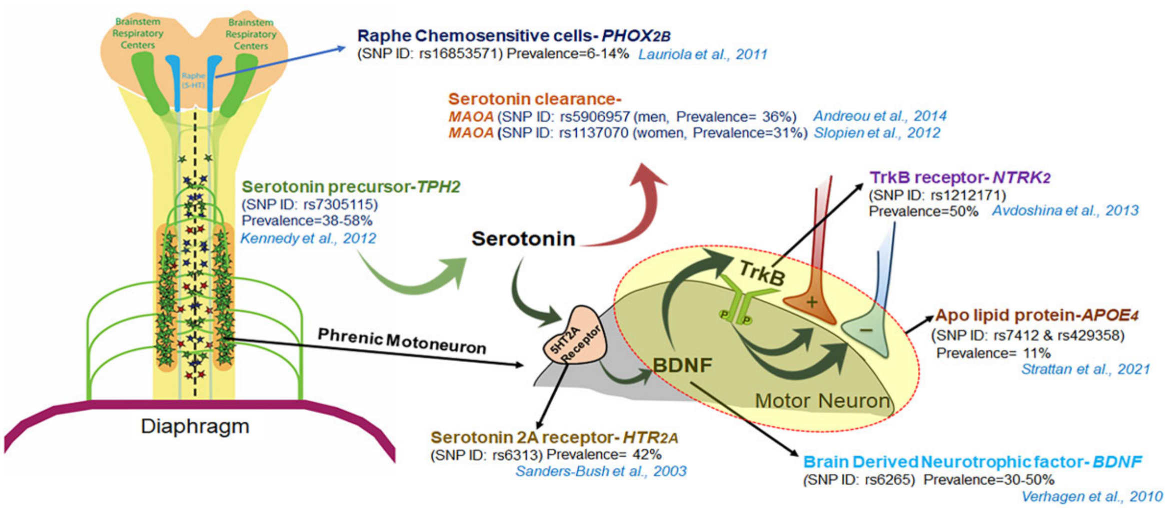


Figure 1. Conceptual diagram depicting cell signaling mechanisms (and candidate biomarker genes) for AIHH-induced respiratory motor plasticity. The panel of SNPs with a population prevalence of >10% were tested for association with reduced AIHH-induced plasticity in humans. These include 6 SNPs in genes 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.

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 Welch et al.[3]). For more information concerning methodological approaches and results, see Supplementary Material and Welch et al.[3]

Participants

Seventeen participants (age range = 20–40 yr, mean age = 27 ± 5 yr, 9 females) signed a written informed consent form to participate in the study.[3] Participants with known pulmonary diseases (eg, asthma), cardiovascular diseases (eg, hypertension, diabetes mellitus, and morbid obesity), respiratory, neurological, or infectious disease/illness, a history of seizures, migraine (in prior 6 mo), and/or metallic implants around the head, chest or shoulder region were excluded from the study. Participant's physical training status and fitness was not considered. Females were screened for pregnancy. Participants were asked to refrain from caffeine consumption 8 h 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 d (separated by ≥3 d): 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% O_2 and 4.5% CO_2 (balance N_2). 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 s (P0.1) 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 percentage change from baseline [(Post − Pre)/Pre × 100].

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

Table 1. Demographics and SNP genotype classification details. Includes individual participants' percentage change from baseline in diaphragm MEP amplitudes and mouth occlusion pressure (P0.1) following AIHH and Sham exposures.

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**)*	Percentage change from baseline in Diaphragm MEP: **AIHH**	Percentage change from baseline in Diaphragm MEP: **Sham**	Percentage change from baseline in P0.1: **AIHH**	Percentage change from baseline in P0.1: **Sham**
S01	40	M	CC	TT	**CC**	APO-E3/E3	CT	**GG**	AA	AA	GG		150.3	130.8	110.2	86.3
S02	29	F	**CT**	TT	**CC**	APO-E3/E3	CT	AG	AA	AG		CC	92.3	74.9	121.4	114.4
S03	28	F	CC	TT	**CC**	APO-E3E3	CT	AG	AA	**GG**		CT	100.9	107.9	116.6	126.9
S04	27	F	**CT**	TT	CT	APO-E2/E3	**TT**	AG	AA	AG		CT	117.3	107.7	131.0	96.5
S06**	24	F	**CT**	TT	**CC**	APO-E3/E3	**TT**	**GG**	AA	AA		CT	215.5	95.9	123.2	78.8
S07	30	M	**CT**	TT	CT	APO-E2/E3	CT	AA	AA	AG	GG		156.0	147.0	86.6	114.8
S08	21	M	**CT**	**CT**	CT	APO-E2/E4	CT	AG	AA	AG	GG		142.7	65.5	145.9	98.1
S09	36	F	CC	**CT**	**CC**	**APO-E3/E4**	CT	**GG**	AA	**GG**		CC	69.3	52.6	105.7	113.1
S10	24	F	CC	**CT**	**CC**	**APO-E3/E4**	CT	AG	AA	AG		**TT**	85.3	127.0	119.3	98.0
S11	32	F	CC	**CT**	**CC**	**APO-E3/E4**	**TT**	AG	AA	AG		CT	91.4	63.6	83.5	125.9
S12	34	M	**CT**	TT	**CC**	APO-E3/E3	CT	AG	AA	AG	GG		137.7	103.8	61.9	75.2
S13	21	M	**CT**	**CT**	**CC**	**APO-E3/E4**	CT	AG	AA	AG	**AA**		110.7	93.4	171.6	109.7
S14	24	M	CC	TT	**CC**	APO-E3/E3	CT	AG	AA	**GG**	GG		106.4	89.9	158.6	98.7
S15	23	F	CC	TT	**CC**	APO-E3/E3	CT	AG	AA	**GG**		CT	94.2	70.3	93.7	69.5
S16	22	M	**CT**	**CT**	**CC**	**APO-E3/E4**	**TT**	AA	AA	**GG**	**AA**		120.2	82.4	193.4	126.0
S17	26	M	CC	TT	**CC**	APO-E3/E3	**TT**	AG	AA	**GG**	**AA**		164.4	87.5	112.6	94.7
S18	31	F	CC	TT	CT	APO-E2/E3	**TT**	AG	AA	**GG**		CC	141.4	73.4	91.1	104.9

**Outlier. Genotype letters in bold indicate a dysfunctional allele.

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 Scientific) and sample purity was estimated by absorbance ratio of A260/A280 (sample range: ≥1.8–2.0). Extracted DNA was diluted to 1 ng/μL 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 Scientific 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, ie, 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"; and (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 *APOE3/4* and impaired AIHH-induced diaphragm plasticity in humans, we performed follow up experiments in adult male Sprague–Dawley rats (345–385 g; Envigo, IN, USA) with homozygous knock-in humanized *ApoE3* (h*ApoE3*; ID #395, $n = 4$) or *ApoE4* (h*ApoE4*; ID #359, $n = 3$). Neurophysiology experiments were performed in urethane anesthetized, paralyzed, and ventilated rats at times consistent with human AIHH treatments (ie, active phase; 12 AM 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] Ethical approval of all experiments were granted by the University of Florida Institutional Animal Care and Use Committee.

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 percentage 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.

Table 2. Association of SNPs with percentage change from baseline in diaphragm MEP amplitudes.

SNP	Estimate	Std. Error	t-value	P-value
BDNFval/met	0.2109	0.1928	1.0938	0.2939
APOE3/4	−0.3802	0.1739	−2.1868	0.0476*
NTRK2	−0.1515	0.1633	−0.9279	0.3703
HTR2A	0.1995	0.1889	1.0559	0.3102
TPH2	−0.2506	0.1234	−2.0312	0.0632
MAOA (male)	0.0239	0.0934	0.2557	0.8084
MAOA (female)	−0.2862	0.3171	−0.9026	0.4015

*$P < 0.05$.

Table 3. Association of SNPs with percentage change from baseline in mouth occlusion pressure in 0.1 s (P0.1).

SNP	Estimate	Std. Error	t-value	P-value
BDNFval/met	0.0212	0.1435	0.1475	0.885
APOE3/4	0.1997	0.1339	1.4908	0.1599
NTRK2	0.0411	0.1196	0.3433	0.7369
HTR2A	0.1109	0.1369	0.8099	0.4325
TPH2	0.0002	0.1009	0.0017	0.9987
MAOA (male)	0.0948	0.146	0.6496	0.5446
MAOA (female)	−0.0133	0.1287	−0.1031	0.9213

*$P < 0.05$.

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 percentage change from baseline. Phrenic responses were analyzed using a 2-way repeated measures ANOVA with Tukey's *post-hoc* analysis (SigmaPlot, v12.0; Systat Software, San Jose, CA, USA). Differences were considered significant when $P < 0.05$. Data are expressed as mean ± SD.

Results

Demographics, genotype, and pre to post percentage 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 percentage change in diaphragm MEP amplitudes and P0.1 are provided in Tables 2 and 3, respectively. 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 percentage change in diaphragm MEP amplitudes (Figure 2). Therefore, data

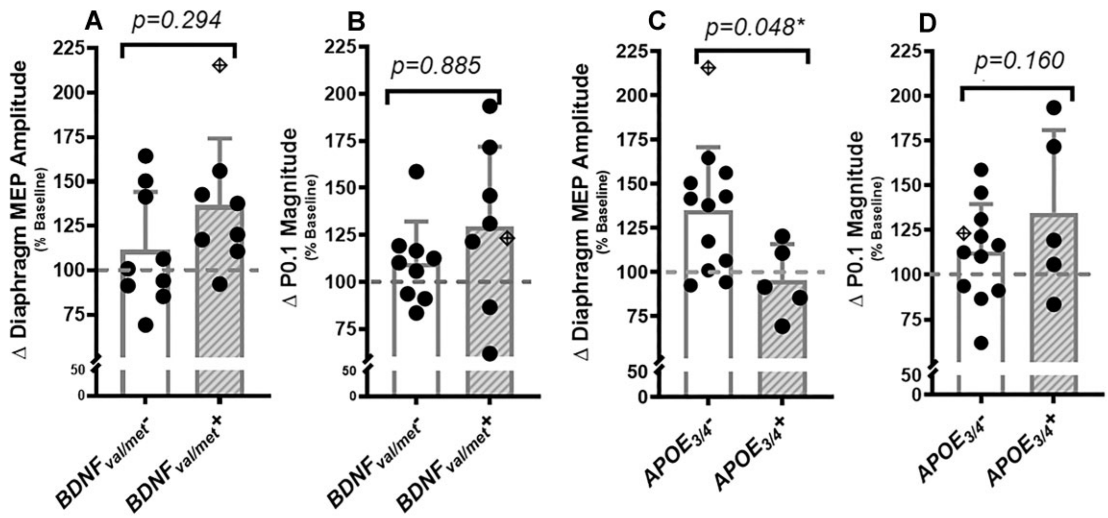


Figure 2. Relative (percentage change from baseline) changes in diaphragm MEP amplitudes and mouth occlusion pressure (P0.1) in individuals with *BDNFval/met* (panels A and B) and *APOE3/4* (panels C and D) SNP. No associations were observed between individuals with *BDNFval/met* and the change in MEP 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 percentage change in diaphragm MEP amplitudes, therefore, the data were not included in group analyses.

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 percentage 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* (ie, *APOE3/4*); none were homozygous for *APOE4*. The *APOE3/4* genotype was associated with diminished percentage change in diaphragm MEP amplitudes following AIHH (Figure 2C, Table 2, $P = 0.048$, $t = -2.187$). The percentage change in diaphragm MEP amplitudes was 38% lower in individuals with *APOE3/4* (*APOE3/4*+) versus individuals carrying other allelic *APOE* isoforms (eg, *APOE3/4*−). In contrast, no significant association between *APOE3/4*+ and percentage 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 percentage 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 percentage change in diaphragm MEP amplitude following AIHH (Figure 4A; $r = 0.08$, 95% CI = −2.47–3.32, $P = 0.758$). 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 = −5.75–4.38, $P = 0.752$). However, males had significantly higher percentage 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 percentage 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 percentage 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 (h*ApoE3*: 0.023 ± 0.007 V; h*ApoE4*: 0.022 ± 0.013 V). On the other hand, AIHH elicited significant phrenic long-term facilitation in h*ApoE3* ($P = 0.025$ versus baseline), but not in h*ApoE4* rats ($P = 0.995$). A significant interaction between genotype and time post-AIHH was observed in phrenic long-term facilitation magnitude (Figure 3B;

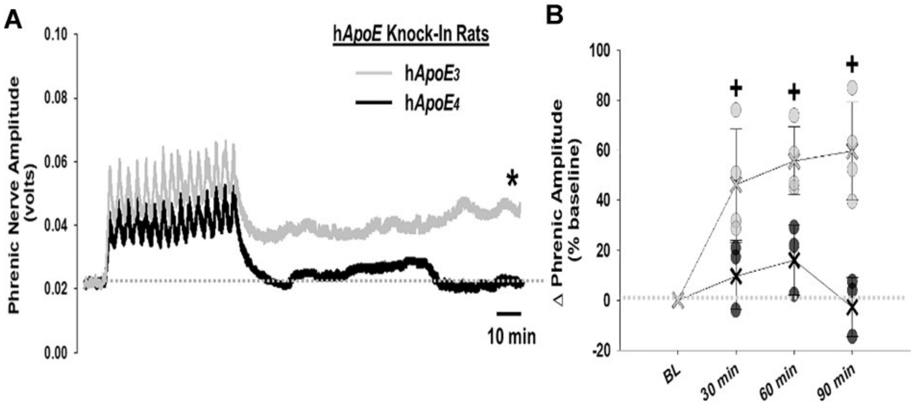


Figure 3. AIHH elicits phrenic long-term facilitation in h*ApoE3* but not hApoE4 knock-in rats. Panel A shows average traces of phrenic nerve amplitude for h*ApoE3* (*n* = 4; gray) and h*ApoE4* (*n* = 3; black) knock-in rats, *P < 0.050 versus baseline. Panel B phrenic burst amplitude (percentage change from baseline) in hApoE3 (gray circles) and h*ApoE4* (black circles) rats, +P < 0.005 versus h*ApoE4*. Δ = change. Results expressed as mean ± SD.

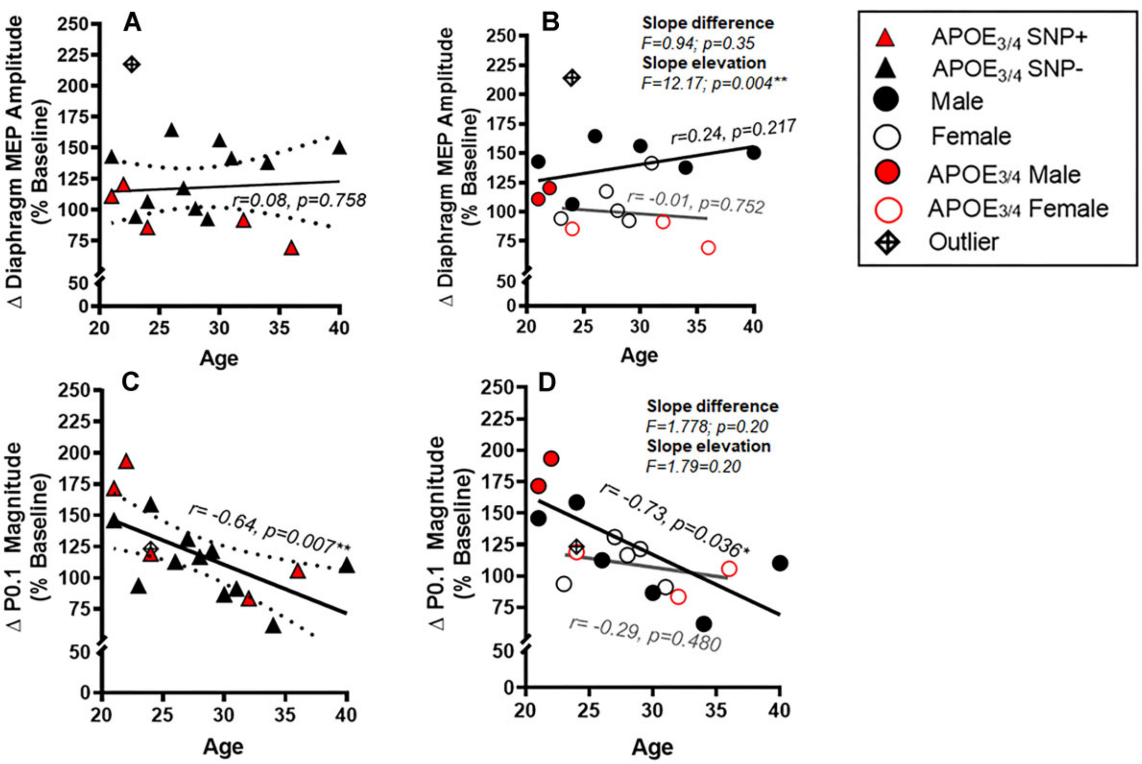


Figure 4. Relationship between age and sex on the magnitude (percentage change from baseline) of change in diaphragm MEP amplitudes (panels A and B), and mouth occlusion pressure in 0.1 s (P0.1, panels C and D) following AIHH. No association between age and the magnitude of change in diaphragm MEP amplitudes 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). 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 percentage change in diaphragm MEP amplitudes, therefore, the data was not included in group analyses.

F = 5.93, P = 0.007). AIHH-induced phrenic long-term facilitation in h*ApoE3* rats was significantly greater than h*ApoE4* at 30 min (P = 0.004), 60 min (P = 0.002), and 90 min (P < 0.001) post-AIHH. While the "*n*" for rat experiments was 3–4 rats per genotype, the power of the performed experiments was 0.823 and the effect size between h*ApoE3* and h*ApoE4* was quite large (Cohen's d = 3.858). Therefore, using additional rats would have violated commonly accepted practices relating to minimizing animal use. Arterial CO_2 partial pressures at baseline (h*ApoE3*: 43.9 ± 1.5 mm Hg; h*ApoE4*: 45.7 ± 1.2 mm Hg) and 90 min post-AIHH (h*ApoE3*: 44.4 ± 1.6 mm Hg; h*ApoE4*: 46.2 ± 0.4 mm Hg) 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 percentage 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 yr), 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 h*ApoE4* and h*ApoE3* knock-in rats using a nearly identical AIHH protocol to humans (15, 1-min episodes of hypercapnic-hypoxia during the night, or the active phase for rats). Whereas rats with h*ApoE3* manifested robust AIHH-induced phrenic long-term facilitation (~60% increase at 90 min post AIHH), h*ApoE4* rats failed to express significant plasticity. Thus, *APOE4* undermines AIHH-induced respiratory motor plasticity in rats. This further strengthens findings reported here in humans, and the need for biomarker identification in clinical trials. Our data support an earlier report by Strattan and colleagues[47] where h*ApoE4* 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 versus 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 h*ApoE4* 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 participants' 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 (ie, 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 yr) 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 s 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[19,66]; (2) diminished serotonergic function[18]; and/or (3) increased extracellular CNS adenosine levels.[67–69]

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,[19] but increases in middle-aged females (when normalized for stage of the estrus cycle).[70] Estrogen suppresses pro-inflammatory microglial activities [71] and even mild inflammation impairs phrenic long-term facilitation.[55,72,73] Testosterone is necessary for phrenic long-term facilitation in males because it is a substrate for aromatase-dependent CNS estrogen formation.[66] In male rats, testosterone peaks at ~2–6 mo of age, equivalent to ~18–40 yr in humans,[74–76] which is then followed by a gradual decline, similar to human males in the ~40–60 yr age range.[77,78] Since the age of our participants ranged from 20 to 40 yr, reduced serum sex hormone levels are unlikely to explain variance in P0.1 responses; furthermore, the percentage 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.[79,80] Although direct evidence of an increase in extracellular adenosine levels in the central nervous system of humans is not available to the best of our knowledge, several convincing animal models confirms this observation,[81–83] potentially explaining reduced P0.1 plasticity with age in our study.

Limitations

This is a proof of principle study to investigate the impact of individual biological factors, such as genetics, age, and sex, on ability of AIH to elicit respiratory motor plasticity in healthy young adults. Our moderate AIHH protocol has not been previously studied in humans; thus, we deliberately chose normal healthy humans to characterize this response, and were not focused initially on the impact of age and sex. Since this work is not a clinical study that reflects the range of age, sex, and genetics, it must be interpreted cautiously as we translate our findings in clinical populations. Follow-up studies targeting a broader age range reflecting a typical population of people with SCI are needed to validate the findings reported here before extrapolating them to clinical trial design.

The menstrual cycle phase in female participants was not controlled in this study. The human menstrual cycle is a complex and dynamic process that involves hormonal fluctuations with associated changes in physiological and psychological function. The timing and intensity of these changes can vary widely between individuals, making it difficult to control for their effects in a small sample size. In the present study, we did not have sufficient statistical power to detect possible menstrual cycle effects. Further, menstrual cycle-related effects on neuroplasticity may be influenced by a variety of factors, including age, contraception use, and underlying medical conditions. Controlling all of these factors will require careful selection of participants in a larger sample size and include additional statistical analyses.

The humans studied here included a number of individuals that were heterozygous for *APOE4*, whereas the knock-in rats studied were homozygous for the human *APOE4* allele. It is certainly possible that hetero- versus homozygosity will impact the results, although other reports suggest that people with either one and two *APOE4* alleles both exhibit worse neurological outcomes, longer hospital stays, and less motor recovery during rehabilitation versus individuals with other *APOE* alleles.[25,84] Nevertheless, studies of rats homozygous for the *APOE4* allele may over-estimate impairment of AIH-induced phrenic motor plasticity (which was abolished) versus rats (or humans) with a single *APOE4* allele.

Future studies comparing homozygous versus heterozygous rats and humans will yield important insights.

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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Supplementary Material

Supplementary material is available at the *APS Function* online.

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Conflict of Interest

None declared.

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

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