Title: Sex and APOE Genotype Influence Respiratory Function Under Hypoxic and Hypoxic-Hypercapnic Conditions

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1

ABSTRACT

Ventilation is regulated across many systems, with respiratory rhythm formation occurring in the brainstem but modulated in response to: chemoreception, respiratory muscle afferents, and volitional control. Alterations within any of these systems may result in disruptions of appropriate respiratory control and ultimately, disease. The apolipoprotein *(APOE)* gene has been studied due to its influence on Alzheimer's disease (AD) development and work in an *APOE* mouse model recently demonstrated impaired respiratory motor plasticity following spinal cord injury (SCI). Additionally, individuals with AD may co-present with obstructive sleep apnea (OSA). Despite the prominence of *APOE* genotype and sex as factors in differing AD and OSA severity, little is known about the impact of these on respiratory control. Using mice expressing two different humanized *APOE* alleles, we characterized how sex and the presence of E3 or E4 alleles influenced ventilation during baseline (normoxia) and during respiratory challenge. We show that sex and genotype influence breathing under hypoxia, which may have clinical implications in the context of AD and OSA. Additionally, female mice, while responding robustly to hypoxia, were unable to recover to baseline respiratory levels, emphasizing sex differences in disordered breathing and potentially indicative of high metabolic cost.

INTRODUCTION

Breathing is a tightly regulated system that incorporates both feedback and feedforward control, however genetic disorders or predispositions can affect respiratory regulation [1,2,3-5]. Within individuals that develop Alzheimer's disease (AD), there is an increased prevalence of specific apolipoprotein *(APOE)* alleles. APOE has been extensively linked to developing cognitive deficits associated with AD however research associated with the impact of *APOE* on respiratory control is limited [6]. In addition to genetic contributions to cognitive decline, respiratory impairments such as obstructive sleep apnea (OSA) can lead to cognitive impairments exacerbating AD symptoms [7]. More than 10% of Americans suffer from moderate to severe OSA with greater occurrence in men [8]. Recent European studies have shown that this number has the potential to be even higher, as much as 23.4% in women and 49.7% in men [9]. Not only do these findings highlight the prevalence of sleep apnea, but they are also indicative of the sexual dimorphism of sleep disordered breathing. In recent years, scientists have continued to address the higher male prevalence of OSA as well as the impact of age on OSA development [10]. In addition to OSA, approximately 1% of the United States population exhibits central sleep apnea resulting from impairments in central chemoreception [11].

Severe respiratory impairments, including congenital central hypoventilation syndrome leading to fatal apneas, can result from genetic mutations[12,13]. It follows that genetic polymorphisms play a critical role in the onset and severity of OSA pathology and symptoms [14,15]. The *APOE* gene encodes for the lipid membrane transport protein apolipoprotein E (ApoE) [16]. ApoE has been extensively classified and studied due to its influence on the development of Alzheimer's disease (AD) in the human population. It is estimated that one *APOE* allele, the ApoE4 allele, results in a 4-fold increased risk of developing AD and homozygous carriers exhibit a 15 fold increase [17]. About one in five people are carriers of the ApoE4 allele. Individuals with AD exhibit severe

cognitive decline and neuroinflammation, which are also hallmarks of OSA. Severe OSA results in frequent sleep arousals (up to 68/hour) and this sleep fragmentation has been shown to increase neuroinflammation and impair cognition [18,19,20]. Imbalances in respiratory control caused by this neuroinflammation have been shown to impair models of respiratory plasticity [21,22], and this impaired respiratory plasticity could limit expected response to hypoxic stimulus. Indeed, our lab has recently demonstrated that there is a sex dependent relationship on spinal motor plasticity in mice expressing the humanized ApoE3 and E4 genes following spinal cord injury (SCI) [23]. In this recent study, female mice expressing the E3 allele maintained diaphragmatic activity following intermittent hypoxia while E4 females had significantly depressed activity. Unlike the female mice, males did not exhibit a difference in diaphragmatic activity based on genotype. To extend these findings, we sought to characterize the relationship between *APOE* genotype and sex in awake mice.

We hypothesized that E4 mice would have less respiratory reserve (i.e., difficultly responding to gas challenges) compared to E3 mice. OSA occurs more frequently in men, thus we additionally hypothesized that there would be differences between the male and female mice in response to hypoxia and hypoxic-hypercapnia in our humanized *APOE* model. To test these hypotheses, we utilized whole body plethysmography (WBP) to measure ventilation in a humanized *APOE* mouse model under normoxia (21% O_2), and two different gas challenges. These challenges were designed to mimic conditions of respiratory stress in OSA by rapidly lowering available oxygen to create a hypoxic environment or by lowering oxygen while adding carbon dioxide to create a hypoxic-hypercapnic environment.

Our results provide a characterization of stress-induced ventilatory responses in humanized *APOE* male and female mice as well as support further investigation into the potential effects of sex and genetics on a multitude of respiratory affiliated disorders. Furthermore, this study highlights the importance of sex and genetics on our biologically diverse human population [24]. Finally, *APOE* genotype and sex findings from this study in the context of experimentally induced hypoxia and hypoxic-hypercapnia are highly relevant to individuals with AD and provide additional framework in our treatment and understanding of respiratory diseases such as OSA. [25,26].

METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Mice expressing human *APOE* isoforms under control of the endogenous mouse *APOE* promotor (targeted replacement mice) were backcrossed for at least 10 generations to the C57BL/6 background [27,28]. Mice were group-housed by sex and genotype on a 12/12 light/dark cycle and fed normal chow diet *ad libitum*. Mice undergoing hypoxic challenge (n=33) weighed 36.9-48.6 g with ages ranging from 367-441 days. Mice undergoing hypoxic-hypercapnic challenge (n=16) weighed 32.02-52.7g with ages ranging from 472-477 days.

	Genotype	n	Average weight (g) (SD)	Average age (d) (SD)
Hypoxia males	E3	9	48.8 (4.7)	410 (16.6)
	E4	8	45.5 (4.6)	441 (65.3)
Hypoxia females	E3	8	36.9 (7.5)	367 (10.1)
	E4	8	37.5 (5.3)	377 (9.0)
Hypoxic-hypercapnia males	E3	4	52.7 (2.8)	473 (0)
	E4	4	47.5 (6.0)	472.5 (4.0)
Hypoxic-hypercapnia females	E3	3	51.2 (5.9)	477 (0)
	E4	5	32.02 (4.2)	476 (3.8)

Table 1. Number, average weight, and average age of mice used in hypoxia and hypoxic-hypercapnia experiments

Hypoxia and hypoxic-hypercapnia experiments

Whole body plethysmography (WBP) (DSI, Buxco FinePointe) was used to measure the ventilatory response during hypoxic (11% O_2 balance nitrogen) and hypoxic-hypercapnic (7% CO_2, 10.5% O_2, balance nitrogen) challenge in adult mice. We extracted respiratory rate (breaths/min), tidal volume (mL/breath/g), and minute ventilation (mL/min/g) from the WBP flow traces. Experiments took place for approximately 90 minutes during daylight hours between 0930 and 1600 with food and water restricted during the 90-minute measurement phase. To ensure consistency across all measures, WBP machinery was recalibrated prior to every trial. Immediately prior to baseline readings, a 40-minute period of in-chamber acclimation occurred to minimize non-ventilatory respiratory behaviors associated with exploring the chamber. Ventilatory measurements following acclimation started with a 30-minute period of normoxia (21% O_2, balance nitrogen), then 10 minutes of respiratory challenge concluding with a 5- or 10-minute period of normoxia. Challenge design can be seen in Figure 1a and the number of animals per group is shown in Table 1. Following trials, mice were returned to their home cage in the vivarium. WBP chambers were thoroughly cleaned and dried between each group.

Data analysis and statistical analysis

The normality and variance of the data was determined to ensure appropriate assumptions were met. If data within a particular experiment did not meet these assumptions non-parametric tests were used. Specific

statistical tests for each figure are listed in the figure legends. Ventilatory measures were calculated in DSI FinePointe software and exported to generate graphs using MATLAB (MathWorks, Version 2021b). For hypoxic ventilatory challenge, hypoxic-hypercapnic ventilatory challenge, and post challenge measures, graphs were normalized as percent change relative to baseline. For statistical analysis, an ANOVA was used for graphical comparisons between sex and genotype. Tukey-Kramer post-hoc comparison was used to compare within variables. Results were considered statistically significant if $p < 0.05$. Investigators were not blinded to genotype or sex.

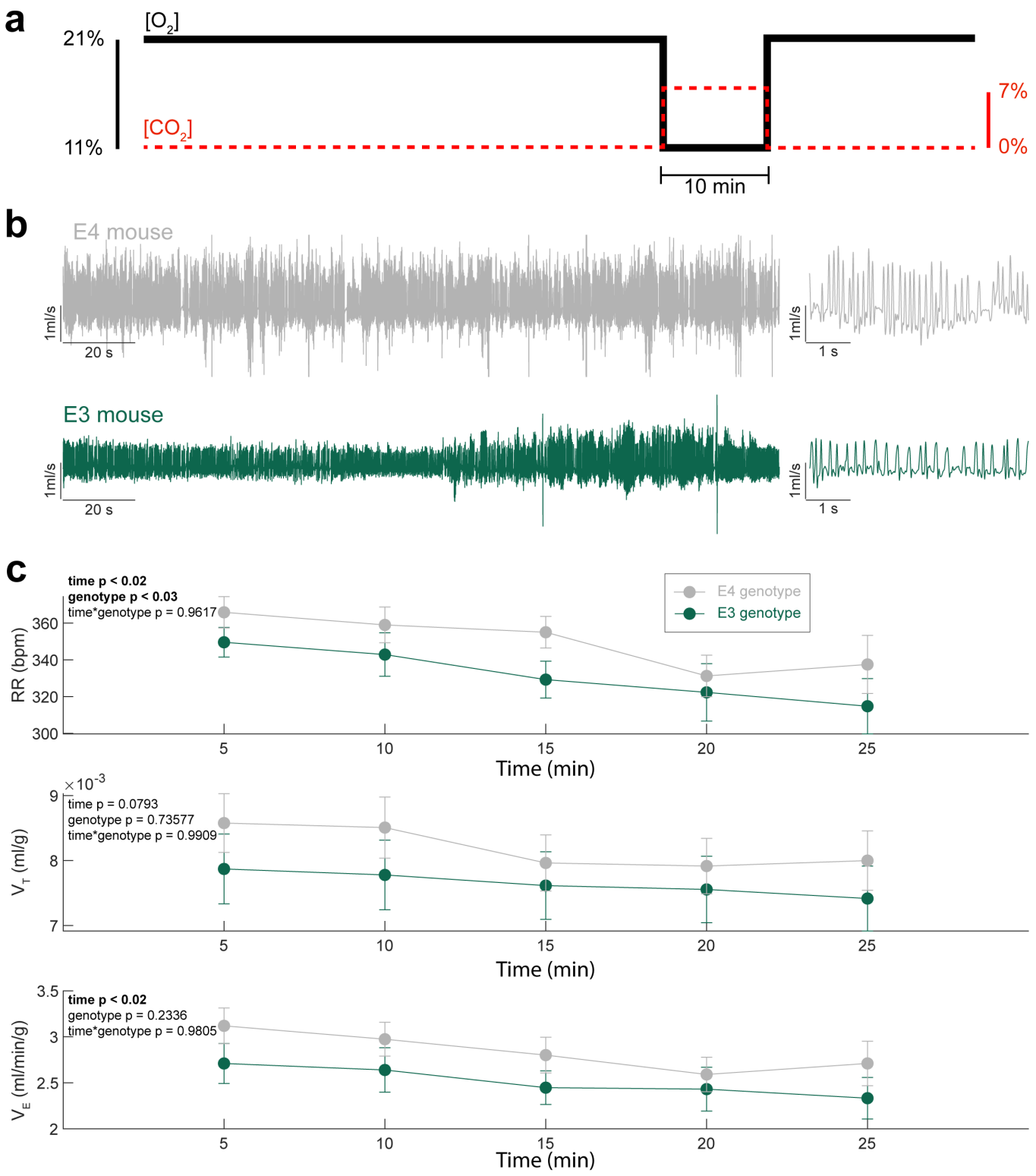

Figure 1. Experimental timeline and comparison of the effect of genotype. a) Timeline of WBP hypoxic and hypoxic hypercapnic challenges. **b)** Plethysmography airflow traces from a representative ApoE3 (green) or ApoE4 (gray) mouse during baseline (normoxia). **c)** Quantification of plethysmography during baseline (normoxia) in five-minute bins, testing for an overall effect of genotype prior to assessing sex differences (Two-way ANOVA mean ± SEM; n= 25 (ApoE4, 13F/12M), n=24 (ApoE3, 11F/13M)).

RESULTS

Female mice breathe harder to maintain baseline ventilatory pattern during normoxia

To determine differences in baseline breathing, ApoE3 and ApoE4 male and female mice underwent a 25-minute period of normoxia prior to a 10-minute respiratory challenge. Figure 2 shows a comparison of sex and genotype at baseline tidal volume, minute ventilation, and respiratory rate. Sex differences were present across all measures. The weight corrected tidal volume of male human transgenic *APOE* mice was lower than the female mice ($p<0.0001$, $F_{(1)} = 24.19$). Additionally, the male mice had a lower respiratory rate ($p<0.02$, $F_{(1)} = 5.98$), and weight corrected minute ventilation ($p<0.0001$, $F_{(1)} = 25.03$). These baseline measures show that there is a sex-dependent effect present that may affect how male and female mice will respond to challenge.

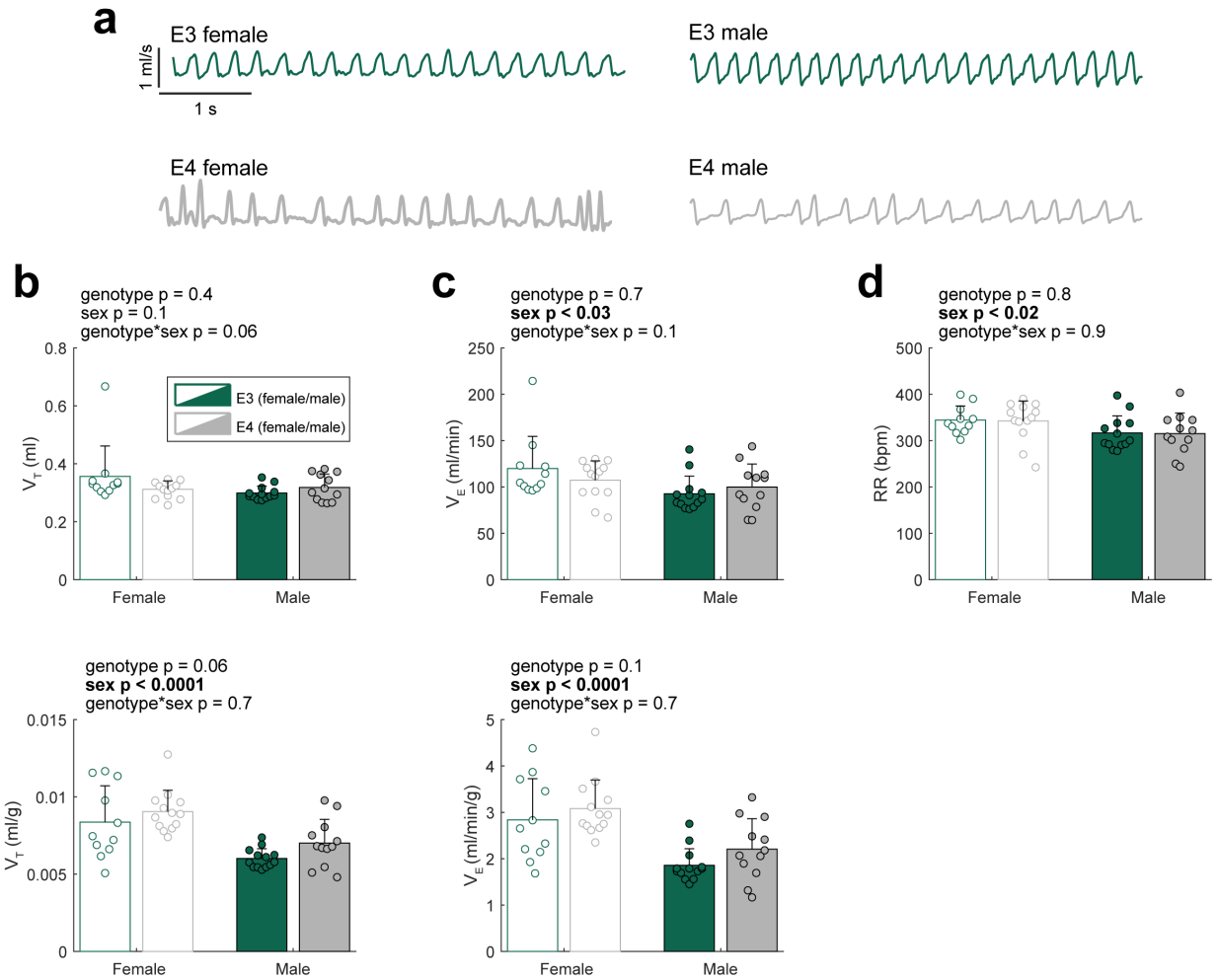

Figure 2: During baseline (normoxic) breathing male mice display lower tidal volume and respiratory rate than female mice. a) Representative airflow traces during baseline (normoxia) from representative ApoE3 and ApoE4 male and female mice. Metrics of ventilation at baseline **b)** tidal volume and weight corrected tidal volume, **c)** minute ventilation and weight corrected minute ventilation, and **d)** respiratory rate. Male mice exhibit lower tidal volume and minute ventilation in weight corrected values, and lower respiratory rate which appears to drive an effect of sex in uncorrected minute ventilation. (Two-way ANOVA, mean + SD; n = 11 (ApoE3 female), n = 13 (ApoE4 female), n = 13 (ApoE3 male), n = 12 (ApoE4 male)).

Female mice initially respond less robustly to hypoxic ventilatory challenge with E4 females maintaining a greater response than E3 counterparts

To further investigate sex- and genotype-dependent respiratory differences, we submitted all sexes and genotypes to a 10-minute hypoxic ventilatory challenge. Figure 3a shows airflow traces from animals undergoing hypoxic ventilatory challenge. Airflow data from all animals in the hypoxia cohort is quantified in Figure 3b, showing the hypoxic challenge period, broken up into two 5-minute bins. Within the first five minutes of hypoxia exposure, there is a clear effect of sex within each measure. During this initial period of hypoxia, female mice had a reduced respiratory rate relative to baseline and male mice had an elevated rate compared to females ($p < 0.0001$, $F_{(1)} = 43.98$). During the final five minutes of the challenge, male mice failed to maintain an elevated respiratory rate compared to baseline, but were still breathing faster than the female mice ($p < 0.002$, $F_{(1)} = 12.69$). All groups increased their tidal volume relative to baseline in the first five minutes of the hypoxic challenge. However, male ApoE4 mice had a less robust response compared to their ApoE3 counterparts ($p < 0.05$, Tukey-Kramer post-hoc), supporting previous work showing that male ApoE4 mice have impaired ventilatory response under hypoxic conditions after SCI[23]. Only the ApoE3 male mice were able to maintain an elevated minute ventilation over the entire challenge period and indeed, the female mice never increased their minute ventilation over baseline.

Post-hypoxia airflow traces show a return towards baseline level (Fig. 3c). Following cessation of hypoxic challenge, there are no significant differences between sex or genotype seen during the entirety of the post-challenge period except for a female genotype difference in the second 5-minute period ($p<0.05$ Fig. 3d.). Interestingly, across all measures, female animals show a decline in tidal volume and respiratory rate relative to baseline, with greater deficit seen in ApoE4 female mice.

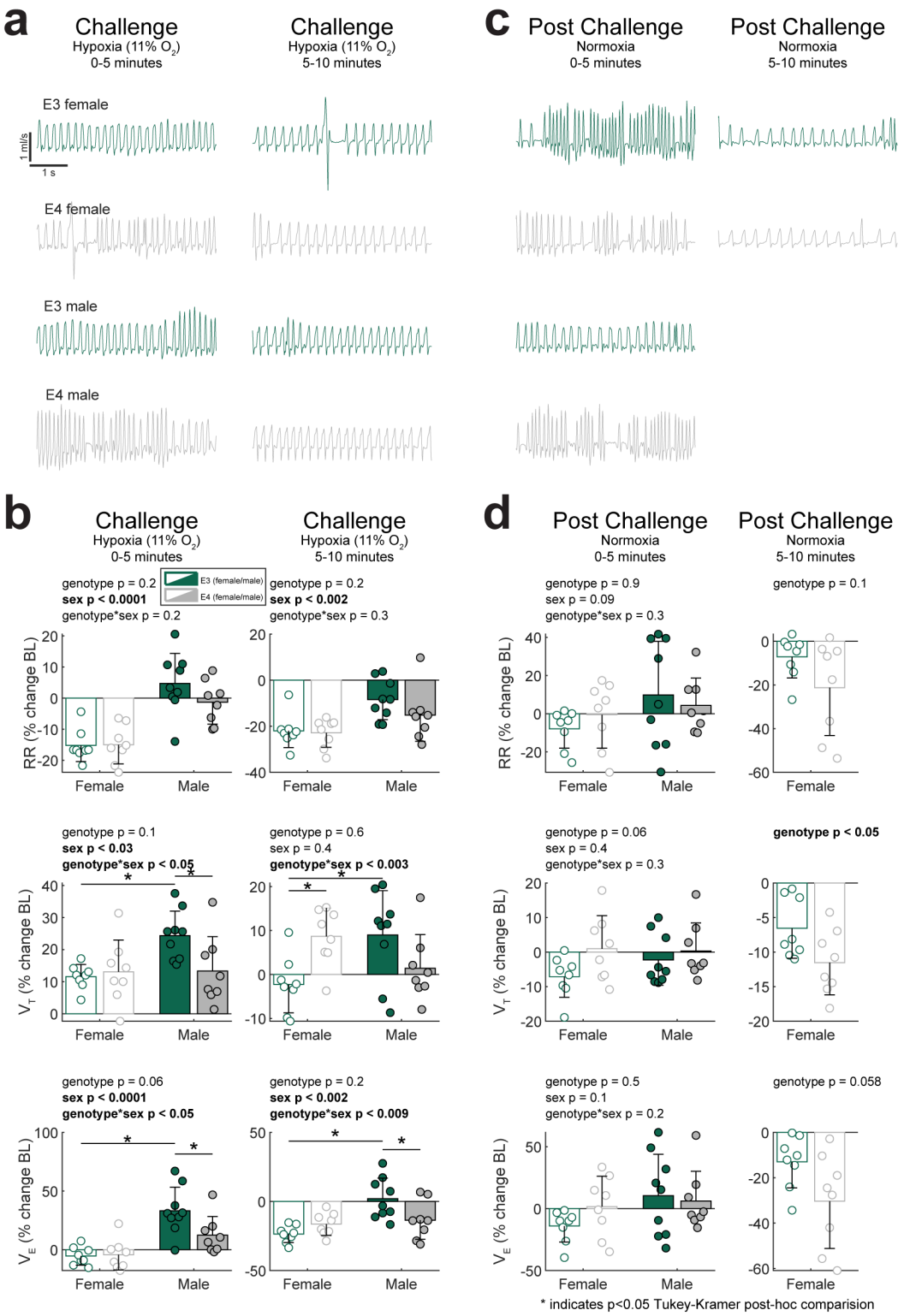

Figure 3: Sex and genotype differences during hypoxic challenge are *APOE* dependent
a) Representative airflow traces during and after hypoxic ventilatory challenge. **b)** Comparison graphs between sex and genotype during hypoxic challenge illustrating the impact of both factors. **c)** Post-challenge airflow traces. **d)** Post-challenge comparison graphs show minimal differences between sex or genotype. Asterisks indicate p<0.05, Tukey-Kramer post-hoc. Two-way ANOVA, mean + SD; n = 8 (ApoE3 female), n = 8 (ApoE4 female), n = 9 (ApoE3 male), n = 8 (ApoE4 male).

Differences in hypoxic-hypercapnic ventilatory response are driven by genotype during challenge, however, following challenge, sex-dependent differences appear

As the male ApoE4 animals during hypoxia did not increase their tidal volume to the same degree as the male ApoE3 animals, we tested for the possibility of a male E4 ceiling effect (i.e., inability to generate larger tidal volume). To test the impact of increased chemosensory stimulation, we exposed a second cohort of mice to a mixed hypoxic-hypercapnic (10.5% O_2, 7% CO_2) challenge. Figure 4a shows a robust hypoxic-hypercapnic ventilatory response (HHVR) across all mice, regardless of sex or genotype.

Figure 4b shows the entirety of the hypoxic-hypercapnic challenge and the corresponding response. There is an effect of genotype in respiratory rate and minute ventilation during the first and second five minutes of hypoxic-hypercapnic challenge. The ApoE3 mice of both sexes had a lower respiratory rate compared to the ApoE4 counterparts during the first five minutes ($p<0.02$, $F_{(1)} = 8.69$) and the final five minutes ($p<0.02$, $F_{(1)} = 8.99$). Minute ventilation mirrors this effect throughout hypoxic-hypercapnic challenge, driven largely by genotypic differences in respiratory rate, indicating that E3 mice have lowered respiratory capacity relative to E4 mice during hypoxic-hypercapnic challenge.

Airflow traces from the period after the hypoxic-hypercapnic challenge (Fig. 4c) show a differential recovery based on sex and genotype. The male mice increased their respiratory rate in the first five-minutes after the challenge, while the female mice maintained the same respiratory rate as during the challenge. The tidal volume of all E3 mice and E4 female mice returned towards baseline, while the tidal volume of the E4 male mice remained elevated.

The post-HHVR (Figure 4d) shows a significant sex-dependent effect throughout the entire 10-minute period across all measures. There is a significant effect of sex on respiratory rate during the first ($p<0.05$, $F_{(1)} = 4.99$) and second ($p<0.02$, $F_{(1)} = 8$) 5-minute periods. Additionally, there was an effect of sex within the tidal volume data in the post-HHVR period in the first ($p<0.009$, $F_{(1)} = 9.73$) and second ($p<0.03$, $F_{(1)} = 6.7$) 5 minutes. As expected, based on tidal volume and respiratory rate, minute ventilation also shows a significant sex-dependent effect in the first ($p<0.007$, $F_{(1)} = 10.86$) and second ($p<0.004$, $F_{(1)} = 13.24$) 5 minutes of post-HHVR. This data may be indicative of impaired respiratory control in the male mice, as they should return toward baseline levels following exposure to hypoxic-hypercapnic conditions.

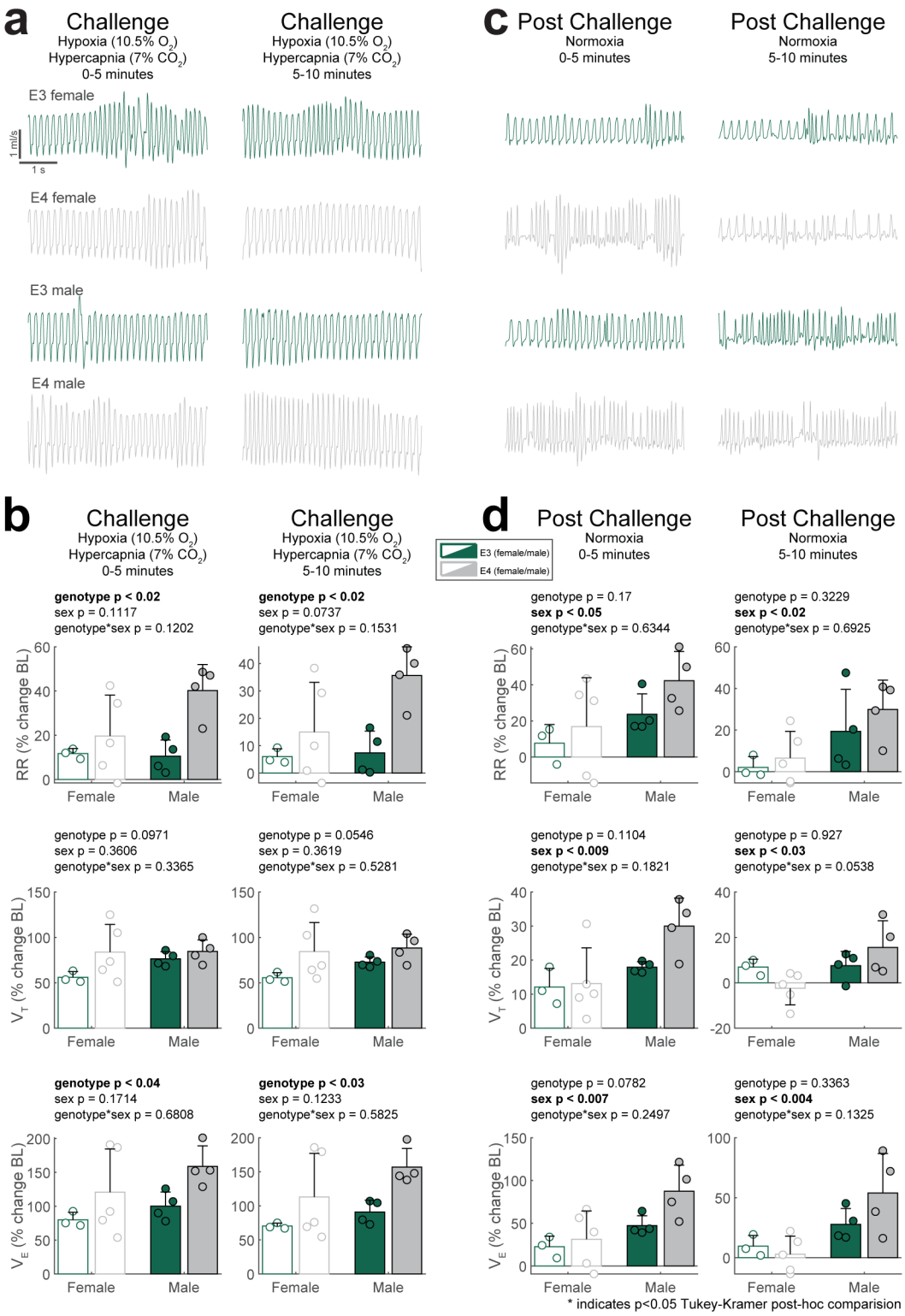

Figure 4: Genotype and sex drive different phases of the hypoxic-hypercapnic ventilatory response **a)** Flow traces show a robust increase in tidal volume during challenge. **b)** Comparison of ventilatory metrics during hypoxic-hypercapnic challenge, considering for effect of sex and genotype. **c)** Representative airflow traces during post-HHVR challenge. **d)** Respiratory metrics during the post-HHVR period. Asterisks indicate p<0.05, Tukey-Kramer post-hoc. Two-way ANOVA, mean + SD; n = 3 (ApoE3 female), n = 5 (ApoE4 female), n = 4 (ApoE3 male), n = 4 (ApoE4 male).

DISCUSSION

This study builds on previous research elucidating the effects of genetic influences and sex on respiratory control [23]. Our results demonstrate that genotype and sex in humanized *APOE* mice plays a role in regulating breathing, specifically under conditions of respiratory stress. In this study we found that under hypoxic conditions, female animals did not increase minute ventilation in the initial period of hypoxia and furthermore displayed decreased minute ventilation as the hypoxic challenge progressed. Male animals increased their tidal volume in the initial phase of hypoxic challenge, however, the E4 males could not maintain this elevation. In contrast, during the hypoxic-hypercapnic challenge, E4 animals were able to increase their respiratory rate beyond E3 counterparts. There were no group differences in tidal volume during this challenge, however, the increased respiratory rate led to an increase in minute ventilation. Across all measures, animals challenged with only hypoxia returned toward baseline levels following challenge. In animals that received hypoxic-hypercapnia challenge, respiratory measures remained elevated.

Following the hypoxic-hypercapnic period, E4 female animals exhibited a similar initial response to their E3 counterparts but maintained this response less effectively. Male animals appear to continue patterns seen during hypoxic-hypercapnic challenge with E4 animals maintaining robust respiratory frequency and tidal volume relative to E3. The differences between hypoxia and hypoxic-hypercapnia are important in the context of pO_2 and pCO_2 chemosensation, respectively. These results are particularly critical when considering the implications of disordered breathing in conditions such as OSA and interrelated neuroinflammation and cognitive impairments.

APOE genotype and the hypoxic ventilatory response

While *APOE* genotype has been evaluated within sleep disordered breathing across broad datasets in human subjects, the research investigating animal models of *APOE* under respiratory stress has been limited [29]. To model an adverse stimulus effect on respiratory pattern between E3 and E4 mice, we utilized hypoxic conditions eliciting the hypoxic ventilatory response (HVR) investigating tidal volume, respiratory rate, and minute ventilation. While there was no effect of genotype on baseline breathing, there was an effect of genotype during hypoxia. Specifically, during the first five minutes of hypoxia exposure, E3 males had a larger increase in tidal volume compared to the E4 males and E3 females. During the second five minutes of the hypoxic challenge, the E4 males and E3 females could not maintain the hypoxic ventilatory response and their tidal volumes decreased towards baseline. The E4 females and E3 males did maintain an increase in tidal volume throughout the challenge, however, the respiratory rate of the E4 males decreased resulting in the lower minute ventilation when compared to E3 males. The inability to maintain elevated tidal volume in the E3 females and E4 males may be indicative of impairments in chemoreceptor function or phrenic-neuromotor control. Chemoreceptor function under hypoxia is susceptible to genetic alterations demonstrated in carotid bodies, although models of humanized *APOE* mice have not been utilized [30]. Future research will elucidate *APOE* specific differences in phrenic motor neuron serotonergic signaling [23].

APOE genotype and hypoxic- hypercapnia elicited respiratory drive

To model the accumulation of CO_2 that coincides with hypoxia during obstructive apneas, mice completed an identical trial with a hypoxic-hypercapnic gas mixture to elicit max chemo-responsiveness. During challenges with hypoxia without concurrent elevated CO_2, E4 male animals displayed an inhibited hypoxic ventilatory response in tidal volume. In contrast, all animals were able to mount a robust initial response during hypoxic-hypercapnic challenge. Tidal volume and minute ventilation increased 50-150% relative to baseline. Additionally, contrasting HVR, E3 and E4 animals exhibited changes in respiratory rate with E4 animals displaying a significantly higher increase in respiratory rate relative to E3 animals. Hypercapnic pCO_2 chemosensation primarily takes place centrally as opposed to peripheral pO_2 sensing carotid and aortic body afferents [31,32]. It could therefore be surmised that while mechanisms of peripheral chemoreception may be impaired in E4 animals, central hypercapnic chemoreception remains unaffected or highly responsive to changes in pCO_2 content. Since respiratory drive remains intact, efferent pathways leaving brainstem respiratory centers may also be unaffected by *APOE*. Finally, on the cellular level, research has shown regional specific effects of ApoE4 on mitochondrial activity and ROS production which may provide another explanation as to why pathways responsive to hypoxia versus those responsive to hypoxic-hypercapnia operate in an inverse manner [33].

Sex differences in humanized APOE mice during hypoxia

Sex differences were found within periods with different inspired gases and respiratory measures. Even in normal breathing, female animals breathed faster and deeper. However, during hypoxic challenge, female animals from both genotypes exhibited a less robust hypoxic ventilatory response than male animals. Tidal volume in response to hypoxia was lower initially in female mice, and the HVR tidal volume was not maintained. Respiratory rate data findings mirror the tidal volume response yet are even more extreme early in HVR. Following hypoxic challenge, female mice elicited no recovery from hypoxic conditions and exhibited continued hypoxic ventilatory decline while male mice returned to baseline levels. These findings are interesting considering that in at least one mouse model, females have been noted to respond more robustly in hypoxic conditions [34]. Sex-dependent responses to hypoxia and hypoxic-hypercapnia have been studied in other animals, although the most relevant background to our current findings was a study in Fischer rats showing that ventilatory response differences are age-dependent as opposed to sex-dependent with middle aged and older female rats having a greater response [35]. Our results contrast this, with male mice responding and recovering more robustly and rapidly than female mice. Future studies should include a cohort of aged matched wild-type male and female controls.

Following hypoxic-hypercapnia sex differences were noted in recovery

Male and female mice of both E3 and E4 background displayed nearly identical hypoxic-hypercapnic responses with changes in ventilation driven by genotype and not sex. However, following the strong

chemosensory response elicited by hypoxic-hypercapnia, female animals rapidly returned toward or below baseline normoxia levels of breathing while male animals continued to maintain elevated respiratory rate and minute ventilation after the inspired gas was returned to normoxic-normocapnic levels. Sex differences in respiratory recovery have been noted previously in neonatal mouse models where female respiratory rhythm generating centers have shown greater ability to recover following a period of severe hypoxia [36]. The data presented here serves to fill a critical gap in knowledge as these *APOE* mice are both closer in relative age and more closely model conditions akin to human sleep apnea physiology [37].

CONCLUSION

This study further clarifies work from our laboratory and others on the effects of genetics, specifically *APOE* on respiratory control, in unfavorable respiratory conditions. Our results show that humanized ApoE4 male mice have more difficulty mounting a response to hypoxic conditions than their E3 counterparts while female mice of both genotypes appear to have difficulty mounting any response in terms of respiratory rate and only E4 females maintain a respective tidal volume increase. Interestingly, E4 female mice have prolonged depression following cessation of hypoxia, not seen in their male counterparts. In hypoxic-hypercapnic conditions, slight genotype differences persist in terms of respiratory rate, however, more interesting perhaps are the sex differences seen in recovery following hypoxic-hypercapnia. Because sleep apnea and disordered breathing have been indicated in earlier onset of disease as well as cognitive impairment this work will serve as a foundation for researching downstream effects of hypoxia and hypoxic-hypercapnia in genotype and sex allowing for the application of these findings to the heterogenous aging human population and to the development of disease [38,39].

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AUTHOR CONTRIBUTIONS

C.E.T.: design of experiment, data collection, data analysis, and interpretation; initial draft of introduction and discussion. L.E.M.: data collection, data analysis, and interpretation; initial draft of methods and results. M.D.S.: data analysis, statistical programming, final graphs; manuscript editing and revisions. J.N.W.: technical support, conceptual feedback. C.M.C.: technical support, conceptual feedback. L.A.J.: mouse model breeding and support, review of manuscript draft. W.J.A.: conception and design of the study, experimental design, interpretation of the data, revision and final approval of the manuscript.

DATA AND CODE AVAILABILITY

The datasets and MATLAB code generated during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL DECLARATIONS

All experiments were conducted in accordance with the NIH Guidelines Concerning the Care and Use of Laboratory Animals and were approved by the IACUC committee at the University of Kentucky.