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EXAMINING HUMAN *APOE* GENOTYPE AND SEX AS MODULATORS OF RESPIRATORY PLASTICITY IN THE PRESENCE AND ABSENCE OF SPINAL CORD INJURY

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

EXAMINING HUMAN *APOE* GENOTYPE AND SEX AS MODULATORS OF RESPIRATORY PLASTICITY IN THE PRESENCE AND ABSENCE OF SPINAL CORD INJURY

There are over 17,000 new spinal cord injuries (SCIs) every year in the Unites States alone. Almost 60% of these injuries occur at the cervical level, potentially leading to loss of function in a variety of sensory and motor systems including upper and lower limbs, respiratory, and autonomics. In addition to the physical and emotional costs, individuals who experience these higher level injuries also face a massive financial burden, incurring over $1 million in expenses during the first year after injury in addition to substantial yearly costs for the rest of their lifetime. A myriad of therapeutic approaches targeting plasticity of spinal circuits have shown efficacy for improving functional recovery in preclinical animal models. However, translation of these strategies has proved less successful, leaving the SCI population with few effective therapeutic options to improve functional outcomes and alleviate the financial toll of their injury. We hypothesized that factors that are present in the human population, but difficult to model in preclinical studies, such as genetic diversity and sex differences, could determine how individuals respond to treatment strategies. Therefore, we investigated how sex and the human alleles of the *APOE* gene (ε2, ε3, and ε4), which encode unique isoforms of the lipid carrier apolipoprotein E (apoE), modulate a well-described form of spinally mediated respiratory motor plasticity that is sufficient to restore breathing function after cervical SCI.

To test whether E4, the isoform of apoE associated with deficits in synaptic plasticity in the brain, also impairs spinally mediated plasticity, we exposed rats to episodic serotonin (5-HT) dosing after they had been treated intrathecally with human apoE3 or E4. IH induces long term facilitation (LTF) of respiratory motor function, which is characterized as a prolonged augmentation of breathing in spinally intact rats. LTF also rescues breathing function in the paralyzed hemidiaphragm following unilateral cervical SCI. Although rats treated with E3 and E4 did not show significant differences in their respiratory response to episodic 5-HT as assessed through diaphragmatic EMG recordings, animals treated with E4 did demonstrate a downregulation of synaptic NMDA receptors. Interestingly, this effect was only present in animals that had received

a cervical SCI. Due to the necessity of NMDA receptor signaling in synaptic strengthening and the induction of LTF, this indicates that human apoE4 may prevent therapeutic strategies from inducing spinal plasticity to mediate functional recovery after SCI.

To increase the clinical relevance of these studies and investigate how sex interacts with *APOE* genotype to modulate respiratory motor plasticity, we also evaluated LTF in male and female targeted replacement *APOE* mice that express the human alleles under the murine promotor. LTF was induced through exposure to therapeutic intermittent hypoxia (IH), which is currently in clinical trials for various functional outcomes in SCI subjects. There were significant effects of both genotype and sex on the respiratory response to IH. Although ε4 was inhibitory to plasticity in males, it was ε3 that proved detrimental in females in the absence of injury. In contrast, injured males showed no significant difference between genotypes while injured ε4 females experienced a decline in breathing activity compared to those expressing ε3.

Together, these results support the hypothesis that sex and genetic background may determine individuals' propensity towards or against plasticity and functional recovery after SCI. By contributing to our understanding of translational barriers for SCI therapeutics, this investigation will inform the development of personalized medicine to overcome these obstacles.

KEYWORDS: Spinal cord injury, Plasticity, Breathing, Genetics, Apolipoprotein E

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DEDICATION

Dedicated to Ethan, Philip IV, Leah, Hannah, Rachael, and Philip V

for your love, support, encouragement, and good humor.

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CHAPTER 1. INTRODUCTION

1.1 Spinal cord injury

Spinal cord injuries (SCI) are life-threatening events that cause permanent disruptions of central nervous system (CNS) physiology, leading to impaired mobility and loss of independence, which dramatically impacts injured individuals' quality of life. Depending on the severity and level of injury, these events can damage both motor and sensory function, as well as autonomic control throughout the body from the neck down. Worldwide there are estimated to be up to 500,000 new SCI cases each year and close to 300,000 people are currently living with an SCI in the United States alone.[1,2] The majority of individuals who sustain SCIs are males and either above the age of 60 or under the age of 30.[1] In America's aging population, an increasing number of injuries, close to 32%, are due to falls, although most result from vehicular accidents.[2] In addition to the physical toll of the injury, SCI is a source of significant financial burden, costing over $1 million in the first year after a high cervical injury.[2] Injured persons whose economic status is below the poverty line have a 62% higher mortality rate than those considered to be above the poverty line.[2,3] Despite decades of research dedicated to SCI research, life expectancy after injury has not increased since the 1980's and there are currently no treatments clinically approved that effectively restore function.[2,4] While numerous experimental strategies have been effective in improving outcomes after SCI in a preclinical animal models, and many have entered clinical trials, few have shown sufficient efficacy in the human SCI population to be successfully translated for widespread clinical use. This indicates that there may be heretofore overlooked or understudied factors in the human population that influence how individuals recover after

injury and how this recovery, whether spontaneous or in response to therapeutics, varies from person to person. Elucidating the role that genetic background and sex differences play in determining the propensity for recovery or the efficacy of treatment strategies in unique individuals may aid us understanding and overcoming translational barriers for SCI therapeutics.

1.1.1 SCI Pathology

SCI is a complex and heterogeneous injury. Over a century ago, it was proposed that spinal cord injury pathophysiology could be divided into primary and secondary insults.[5] The primary injury consists of the initial event that damages the tissue of the spinal cord and disrupts the neural circuitry housed within. The spinal cord is a crucial bidirectional relay between the brain and the periphery, transmitting signals that supply the brain with sensory information, as well as carrying inputs for the control of motor and autonomic functions. In addition to functioning as a relay, the control of many crucial physiological functions and reflexes also originates in the cord, which contains circuits and central pattern generators that are distinct from those located supraspinally[6–9]. Any mechanical force that damages these pathways can lead to motor and sensory deficits. However, this initial trauma is not the only concern for people who sustain an SCI. Following the primary injury, a cascade of events wreak havoc on the damaged spinal cord tissue. Hypoxia due to a loss of blood flow, excitotoxicity from excessive glutamate release, activation and infiltration of inflammatory cells, mitochondrial dysfunction, and the production of oxidative compounds lead to additional cell death.[10–13]

The depletion of both neuronal and non-neuronal cells, along with axonal demyelination and Wallerian degeneration lead to cavitation of the spinal cord at the lesion epicenter.[14–17] Axonal and myelin debris, cytokines, and oxidative stress trigger the activation of glial cells hours after injury.[18,19] In an attempt to aid in wound repair, astroglial processes hypertrophy and overlap, forming a barrier around the lesion cavity.[20] Astrocytes also upregulate production of chondroitin sulfate proteoglycans (CSPGs) and other components of the extracellular matrix that contribute to formation of the glial scar. Injury also stimulates peripheral monocyte-derived macrophage infiltration of the spinal cord. These cells accumulate with CNS-resident macrophages in the lesion cavity.[21] Macrophages then recruit perivascular fibroblasts to accumulate in the lesion site.[22] By 7 days post injury, the lesion core is filled with a fibronectin matrix secreted by these fibroblasts, forming a fibrotic scar in the lesion core that is encompassed by the glial scar.[23,24]

In addition to the astrocytes and fibroblasts that accumulate at the lesion site after injury, immune cells from both the CNS and the periphery also contribute to secondary the injury, scarring, and tissue repair. Microglia are the resident immune cells of the central nervous system. In the uninjured CNS, microglia dynamically function as sentinels, extend and withdrawing their processes in a matter of minutes to monitor their surroundings. These processes are capable of engulfing components of the surrounding tissue and transporting them back to the soma.[25] Microglial processes monitor synaptic functioning by directly contacting synapses in response to neuronal activity, which aids in their crucial role of synaptic pruning during development and after injury.[26,27] Upon CNS insult, microglia are activated and polarized into M1 or M2 phenotypes, which are

classically considered pro-inflammatory or anti-inflammatory, respectively, in resemblance to macrophage classification. M1 macrophages and microglia secrete reactive oxygen species and cytokines such as IL-12, IL-1β, and IFNγ which contribute to tissue damage after injury.[28,29] Conversely, the cytokine expression profile of cells with an M2 phenotype primarily consists of anti-inflammatory molecules including IL-10, IL-13, and IL-7, which encourage neuron growth.[28,29]

In recent years, the accuracy of classifying macrophages and microglia in a strict M1 or M2 dichotomy has been called into question. After CNS injury, macrophages and microglia in the lesion penumbra simultaneously express both M1 and M2 markers and the expression of these molecules changes over time, indicating that their response to injury is too complex and dynamic to be characterized dichotomously as either pro- or anti- inflammatory.[30] One such example of how these terms can be misleading is in reference to the cytokine TNF-α. Historically, TNF-α has been considered pro-inflammatory. Evidence shows that TNF-α damages mitochondria, leading to reactive oxygen species production, and ultimately causing apoptotic or necrotic cell death.[31,32] However, TNF-α also plays an important role in synaptic plasticity. TNF-α signaling stimulates an upregulation of synaptic AMPA receptors and strengthens excitatory synapses, demonstrating that it has divergent functions in cytotoxicity and protection of synaptic integrity.[33,34] Indeed, the inflammatory milieu initiated by SCI may be a driver of secondary tissue damage, while also presenting a potent and dynamic process that could be harnessed to enhance plasticity, and subsequently, recovery.

1.1.2 Assessment of Injury Severity

Both primary and secondary injury contribute to the overall severity of an individual's SCI. Within the first 72 hours after injury, clinicians assess the extent of the neurological injury using the American Spinal Injury Association Impairment Scale (AIS). The AIS identifies the neurological level of injury (NLI) and evaluates both sensory and motor function below this level. The NLI is identified as "the most caudal segment of the spinal cord with intact sensation and antigravity muscle function".[35] The injury is then designated as "complete" if there is no sparing of sensory or motor function at the sacral spinal cord. Complete injuries receive an AIS grade of A. Otherwise, the injury is deemed incomplete and given a grade of B-E, depending on the amount of motor and sensory function preserved caudal to the NLI. An AIS grade E represents normal sensation and motor function.[35] In people who experience a complete injury, a zone of partial preservation may be observed. This refers to dermatomes and myotomes caudal to the NLI that retain partial innervation.[36]

Over time after the initial injury, individuals' AIS grade can be re-evaluated to measure spontaneous rate of recovery and is used as a outcome measure in clinical trials for SCI treatment strategies.[37–39] The majority of people with injuries of AIS grade B and C improve by one AIS grade by 6 months post injury without any intervention. However, only a third of people with AIS A injuries changed classification by 6 months and only 10% of AIS D patients show improvement in AIS grade during this time.[39] Some concerns over the utility of the AIS have been raised due to its emphasis on sparing of sensorimotor function at the sacral levels, which could distract from the overall neurological severity and consequently diminishes the reliability of the scale as an

outcome measure.[39] In fact, the design of the AIS allows it to reflect an increase in grade despite functional deterioration and vice versa.[40]

Additional assessment of injury severity include quantitative sensory testing, in which vibratory and thermal stimuli are applied to dermatomes caudal to the NLI in order to evaluate the function of the dorsal columns and spinothalamic tract, respectively. This testing provides a more quantitative analysis of sensation than the AIS sensory score.[41] Conduction in the spinal cord can also be measured by electromyography (EMG) or motor-evoked potentials, approaches that are advantageous due to their efficacy in unconscious patients. These evaluations of descending motor tracts are predictive of functional outcome, allowing clinicians to tailor treatment plans for individuals in order to maximize their functional improvement.[42,43] In addition to sensorimotor assessments, it is also crucial to monitor impairments of the autonomic nervous system. Exposure to stimuli that cause vasoconstriction leads to an increase in blood flow to the skin that can be measured above and below the NLI. Since vasoconstriction is controlled by the sympathetic nervous system, vasomotor reflex responses can provide an indication autonomic damage after SCI.[44]

An emerging strategy for assessing severity and predicting outcomes after SCI is the discovery and classification of biomarkers. Biomarkers can be sampled in a variety of ways, with recent studies evaluating imaging techniques or collection of serum or cerebrospinal fluid (CSF). T2-weighted MRI provides visualization of grey versus white matter, allowing for the detection of compression, which is predictive of motor and sensory deficits in non-traumatic SCI.[45] Data published by Brian Kwon and colleagues demonstrates the potential of measuring protein levels of IL-6, IL-8, and GFAP, as well

as certain subsets of microRNAs in CSF to indicate current AIS grade and provide a prognosis for AIS improvements over time.[46,47] Similarly, GFAP and neurofilament protein concentrations in serum also have diagnostic value in predicting SCI severity and outcomes.[48,49] Analysis of these biomarkers associated with specific AIS grades or injury severities has potential to aid the assessment of patients who cannot be graded on the AIS due to unconsciousness, damage to other body systems, or other issues of noncompliance. Accurate biomarkers could therefore contribute to accurate characterization of injuries for enrollment in SCI clinical trials and provide additional outcome measures for therapeutic efficacy.[50]

1.1.3 Supportive Care After Injury

There are currently no FDA-approved drugs that are recommended as standard of care for the treatment of spinal cord injury. However, first responders and clinicians can take action to minimize damage to the spinal cord tissue and prevent disturbances in other body systems. One of the first decisions that must be made is whether to perform surgical decompression to relieve pressure on the spinal cord and if so, when. In cases of central cord syndrome or other stable or non-traumatic spinal cord injuries, spinal decompression may not always be necessary due to the high likelihood of spontaneous improvement.[51] However, in many cases of traumatic SCI, surgical decompression is recommended within the first 24 hours after injury due to its correlation with greater improvements in neurological outcome or AIS grade.[52] Non-surgical decompression using spine traction, in which weight is applied to the skull in order to pull it away from the shoulders and lengthen the cervical spine, has also proved efficacious for some cervical level injuries.[53]

In the first day after injury, SCI patients are at risk for hemodynamic instability due to autonomic dysreflexia or loss of sympathetic tone during neurogenic shock. Compression of the spinal cord can also prevent blood flow to the lesion penumbra, creating a hypoxic state. In order to prevent hypoperfusion, hypotension, and bradycardia, guidelines in the last decade have recommended that mean arterial pressure (MAP) is maintained at 85-90 mmHg during the first week post injury.[54] This aggressive maintenance of MAP is associated with enhanced neurological outcomes measured by AIS grade conversion, walking ability, and bladder function.[55,56] Unfortunately, administration of vasopressors comes with risks that must also be considered. Use of common vasopressors cause complications including tachycardia, bradycardia, atrial fibrillation, and even elevations in troponin, a biomarker of heart damage.[57] Recent research in humans and animal models has explored when and to what extent MAP should be elevated after injury.[58–60] Even chronically after injury, the spinal cord tissue caudal to the injury can remain in a state of hypoxia, limiting the potential for functional recovery.[12]

A major concern for individuals with impaired mobility and sensation after SCI is the development of pressure ulcers. The second most prevalent cause of death after SCI is septicemia, often as a result of epidermal ulceration.[61] Pressure ulcers develop in response to prolonged pressure on the skin, especially over bony areas, which results in a period of ischemia. Numerous studies have examined the impact of body positioning while patients are in supine or prone recumbency on the development of pressure ulcers.[62,63] While body positioning techniques can be used to reduce pressure on the skin overlying the sacrum, trochanters, and hips, an additional approach is to develop a

schedule for regularly turning patients in bed.[64,65] This redistributes pressure and restores blood flow to areas that are at risk for developing ulcers. Similarly, once quadriplegics or paraplegics are maintaining a sitting position for extended periods after injury, pressure relief maneuvers, such as leaning forward, reclining, and doing push-ups must be performed regularly to reduce the risk of developing ulcers.[66–68] However, multiple factors, including individual skin physiology, age, weight, and response to stress must be taken into consideration, emphasizing the need for personalized care in the SCI field.

For people who sustain an injury at or above the T6 spinal level, preventing episodes of autonomic dysreflexia (AD) becomes a constant concern beginning a few months after injury. Below the level of injury there is a disruption in the regulation of preganglionic sympathetic neurons, which reside in the intermediolateral nucleus of the spinal cord between the first thoracic and second lumbar level.[69] The sympathetic division of the autonomic nervous system is crucial in the control of hemodynamics.[70] After injury, sympathetic tone below the level of injury becomes independent of supraspinal control, while above the lesion it is still governed by brainstem inputs. Unperceived noxious stimuli including hyperextension of the bowel or bladder, ill-fitting clothes or shoes, pressure ulcers, or skin lacerations caudal to the injury activate the sympathetic nervous system.[71] The sympathetic “fight or flight” response instigates vasoconstriction to increase arterial blood pressure, which is sensed by baroreceptors of the carotid sinus.[71,72] Baroreceptor feedback to the medullary nucleus solitarius initiates a conversion from sympathetic to parasympathetic dominance to slow the heart rate and decrease blood pressure. However, sympathetic neurons below the NLI that lack supraspinal input remain uninhibited and continue to maintain vasoconstriction, leading

to systolic arterial pressures as high as 325mmHg.[73,74] If left untreated, AD puts patients at risk for stroke, cardiac arrest, seizures, and death.[75]

Maladaptive plasticity in the spinal cord after injury may cause hypersensitivity and enhanced nociception, increasing the likelihood of a stimulus being perceived as noxious.[76,77] Since the stimuli initiating AD are unperceived by the patient due to deficits in sensory function, episodes of AD can happen multiple times a day. They are characterized by sweating, headache, stuffy nose, and flushing above the level of injury.[71] Once AD has been triggered, the most effective way to terminate it is by identifying and removing the stimulus. However, AD is also be treated pharmacologically with nitroglycerine paste or an L-type calcium channel blocker, which relax smooth muscle to alleviate vasoconstriction.[78,79]

1.1.4 Clinical trials for SCI interventions

Although there are currently no FDA-approved drugs for the treatment of SCI, a search on clinicaltrials.gov produces over 800 clinical trials for spinal cord injury, including 427 that have already reached completion. Many of these trials aim to alleviate a variety of comorbidities associated with SCI including neuropathic pain, cardiovascular disease, sleep apnea, and loss of muscle mass. Other trials are focused on the translation of drugs or other therapeutic approaches that have shown potential for improving motor and sensory function to enhance independence and quality of life. Representative examples of currently ongoing clinical trials for SCI that target a variety of complications that can result from the injury are presented in Table 1.

Table 1.1

Target	Intervention	ClinicalTrials.gov Identifier	Principle Investigator
Neuropathic pain	Capsaicin patch	NCT02441660	Michelle Trbovich
Cardiovascular	Systemic hypothermia	NCT02991690	Alan D. Levi
Spasticity	Intrathecal Baclofen	NCT02903823	Peter Kondrad
Maintenance of muscle mass	Testosterone	NCT01652040	Ashraf Gorgey
Preservation of bone mass	Zoledronic acid	NCT01642901	Christina V. Oleson
Sleep Apnea	Episodic hypoxia	NCT02922894	M. Safwan Badr
Inflammation	MT-3921	NCT04096950	Mitsubishi Tanabe Pharma Development
Motor/Sensory functional improvement	Riluzole	NCT01597518	Michael Fehlings
	Lexapro (selective serotonin reuptake inhibitor)	NCT01788969	Thomas G. Hornby
	FAB117-HC (human adipose derived mesenchymal stem cells)	NCT02917291	Ferrer Internacional S.A.
	Epidural stimulation	NCT04123847	Susan Harkema
	Rehabilitation/Training	NCT03504826	Emily J. Fox

In addition to these experimental treatments, many clinical trials have also tested the efficacy of neuroprotective drugs that could reduce secondary injury. Minocycline, an antibiotic which reduces apoptosis of oligodendrocytes and microglia, was proved safe and feasible in a Phase II trial[80] and is now being investigated in an ongoing Phase III trial (NCT01828203). Inhibition of endogenous opioids after injury has also shown neuroprotective effects, leading to the development of clinical trials for opioid blockers such as naloxone and thyrotropin-releasing hormone.[81,82] These antagonists have shown preclinical efficacy in dampening excitotoxicity, preventing ischemic damage, and improving functional recovery after SCI, although results from clinical trials were less conclusive.[81–83]

Importantly, the only drug ever approved for acute SCI was also a neuroprotective agent. Methylprednisolone sodium succinate (MP) is a glucocorticoid with antioxidant capabilities that showed efficacy in preventing lipid peroxidation and neurofilament degradation, while also improving blood flow to the injured cord. [84,85] In a series of three clinical trials known collectively as the National Acute Spinal Cord Injury Studies (NASCIS I, NASCIS II, NASCIS III), various dosages of MP were tested at different time points after injury for a duration ranging from 1 day up to 10 days. Results from these studies demonstrated that high dose MP administered within 8 hours of injury resulted in improved sensory and motor recovery at 6 weeks and 6 months.[82,86,87] However, subsequent criticism of the studies' analyses and concerns over gastrointestinal side effects and increased infection risk led to removal of MP from standards treatment of SCI in the United States.[88,89]

1.1.5 Experimental approaches for preserving and restoring function after SCI

A plethora of diverse experimental therapeutic approaches have been evaluated for their potential to preserve or improve function after SCI in preclinical animal models and in clinical trials. For simplicity, these techniques can be divided into the categories of: neuroprotection, those enhancing plasticity, and those promoting regeneration. In addition to these unimodal methods, combinatorial approaches have also been developed that target both the preservation of existing tissue and the cultivation of new axon growth and synaptic connectivity.

As described in section 1.1.2, initial damage caused by an SCI leads to subsequent secondary injury events including scarring, inflammation, oxidative stress, mitochondrial dysfunction, and axon demyelination and degeneration. Neuroprotective strategies are those that aim to mitigate or prevent secondary injury cascades in order to preserve tissue and functional neural circuitry in the spinal cord. Some neuroprotective compounds have already entered into clinical trials, including MT-3921 and Riluzole, seen in Table 1. Indeed, the only drug that has been approved by the FDA for the treatment of SCI was the neuroprotective corticosteroid methylprednisolone.[82,87] These trials also investigated other neuroprotective agents naloxone and tirilazad, both of which were less effective in restoring function. While later clinical guidelines advised against the use of MP, these guidelines remain a subject of contention for clinicians.[90] Regardless, MP's ability to improve functional recovery emphasizes that neuroprotective agents are powerful tools in the search for SCI treatment strategies.

It was once thought that damaged axons in the CNS were incapable of regeneration due to inhibitory properties of the CNS environment.[91,92] Indeed, in 1981, David and Aquayo proved that injured spinal cord axons are capable of elongating over long distances when the CNS environment is replaced by a peripheral nerve graft.[93] This seminal discovery led to decades of research dedicated to finding treatment strategies that create a CNS microenvironment that is more conducive to axon growth. In addition to peripheral nerve grafts, growth permissive environments have been inserted into the spinal cord in the form lab-manufactured scaffolds of various materials.[94] Therapeutic approaches also include amplifying the presence of neurotrophic factors such as BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3), or GDNF (glial cell-derived neurotrophic factor) to activate genes associated with regeneration.[95,96]

Instead of increasing growth-promoting molecules in the CNS microenvironment, other strategies remove molecules that are inhibitory to outgrowth. A promising therapeutic target is the degradation of chondroitin sulfate proteoglycans (CSPGs), which are found in the perineuronal net surrounding CNS neurons. CSPGs are upregulated following SCI, creating a barrier around the lesion site that halts axon elongation and entraps regenerating end bulbs.[97,98] Degradation of CSPGs using the bacterial enzyme chondroitinase-ABC (ChABC) removes this inhibitory boundary and permits axon outgrowth and regeneration through the injury site, leading to new functional synaptic connections.[99] Further studies have also shown the efficacy of interfering with interactions between CSPGs and their neuronal receptors.[100,101]

Ablation of reactive astrocytes, mediators of glial scar formation after SCI, can also create a growth-permissive state at the cost of exacerbating the inflammatory response to injury.[102,103] However, some of these immune responses to injury can actually stimulate axon growth. For example, macrophage-mediated release of oncomodulin promotes optic nerve regeneration.[104,105] Despite some beneficial effects acutely, sustained immune activation leads to neuronal and glial cell death, making it difficult to modulate inflammation to stimulate favorable responses while avoiding detrimental outcomes.[106] In the 1980's, Schwab and colleagues realized that the myelin produced by oligodendrocytes in CNS white matter also acts as a nonpermissive growth substrate and neutralization of myelin membrane proteins promotes corticospinal tract regeneration through an SCI.[107–109] Since these discoveries, approaches that knockout or otherwise inhibit receptor binding of the myelin proteins Nogo, MAG (myelin associated glycoprotein), and OMgp (oligodendrocyte myelin glycoprotein) have shown efficacy in stimulating both regeneration of injured axons and sprouting of spared fibers.[110–113] Altogether, studies manipulating the injury milieu have provided valuable evidence for the competency of CNS axons to regenerate when neuron-extrinsic barriers to growth are removed.

In contrast to approaches that aim to remove inhibitory factors from the microenvironment to promote axon elongation, others modulate neural intrinsic factors to stimulate the neuronal growth machinery. In a pivotal 2008 study, Park et al. found that deletion of cell growth control genes significantly impacted cell survival and axon regeneration. Specifically, deletion of the tumor suppressor gene PTEN (phosphatase and tensin homolog) promoted axon growth well beyond an optic nerve lesion.[114] This effect

has also been reproduced in the injured spinal cord, even when PTEN is only transiently inhibited by an antagonist peptide or when downstream targets of PTEN are directly activated.[115–117] Axon regeneration is an energy-intensive process, requiring transportation of mitochondria to the growth cone.[118,119] Inhibition of PTEN activates genes associated with mitochondrial transport and enhanced regenerative capacity, indicating that mitochondrial motility may be a key intrinsic factor that influences axon regeneration.[119,120] In a similar manner to PTEN, expression of a GTPase known as RhoA is also implicated in preventing axon elongation.[121] RhoA expression is upregulated after SCI, leading to downstream increases in inflammation and growth cone to collapse, effectively preventing neurite outgrowth.[122] The FDA-approved non-steroidal anti-inflammatory drug ibuprofen suppresses Rho signaling and promotes axonal sprouting.[123] Another fascinating, yet paradoxical way to stimulate the growth machinery of spinal axons is through a peripheral conditioning lesion.[124] Lesioning the peripheral branch of dorsal root ganglion neurons activates signaling pathways that promotes regeneration of dorsal column axons after SCI.[125]

Although regeneration and sprouting of CNS axons may be considered a form of structural plasticity, henceforth plasticity will refer to alterations in the number or strength of synaptic connections. While CNS plasticity will be discussed in greater detail in section 1.2.3, some therapeutic approaches that target spinal plasticity in the context of SCI will be addressed here. Following SCI, spinal plasticity can either be beneficial, leading to functional improvement, or maladaptive, often exacerbating spasticity, pain, and autonomic dysreflexia. One way to combat maladaptive plasticity is through training and rehabilitation strategies, which can reduce spasticity and normalize spinal reflex

firing.[126,127] During such training and exercise protocols, afferent inputs in the spinal cord modulate synaptic plasticity through BDNF-dependent mechanisms to improve motor function after SCI.[128–130]

Neural excitability in the spinal cord can also be modulated through epidural stimulation, which has shown efficacy in improving both locomotor and autonomic function following SCI.[131–135] In epidural stimulation protocols, electrodes are placed epidurally over the targeted spinal segments while a pulse generator is implanted in the abdominal wall.[133,136] A less invasive strategy that enhances plasticity of spinal circuits is intermittent hypoxia (IH). IH consists of exposing subjects to bouts of low inhaled oxygen (hypoxia) interspersed with periods of normoxia or hyperoxia, which activates serotonergic (5-HT) neurons in the raphe.[137] These 5-HT fibers extend throughout the spinal cord, allowing them to modulate the function of various motor systems.[138] Clinical studies have demonstrated improvements in breathing, locomotion, and upper limb function after exposing spinal cord injured subjects to IH.[139–143] The functional improvements that result from IH and epidural stimulation can be further augmented by combining them with task-specific training.[136,144] All of these therapeutic strategies are currently under investigation in clinical trials (Table 1.)

Some therapeutic approaches and combinatorial treatment strategies target multiple mechanisms of neuroprotection, regeneration, or plasticity. In preclinical and clinical investigations, transplantation of a variety of cell types has been of interest due to the cells' ability to create a protective and pro-regenerative microenvironment.[145] Transplantation strategies utilizing neural stem cells, bone marrow-derived stromal cells,

or mesenchymal stem cells have proven effective preclinically in decreasing lesion cavity size and enhancing the amount of spared tissue after injury.[146–149] They can also prevent the spread of inflammation throughout the spinal cord.[150] Neural progenitor cell grafts are capable of forming synaptic connections with host axons to create relays through an SCI lesion site in monkeys.[151] Foundational work from the Bunge lab emphasized the power of Schwann cells in reducing lesion cavitation, promoting axon regeneration, and remyelinating fibers to restore axon conduction.[152] Combining cell grafting techniques with additional strategies such as ChABC-induced PNN degradation, scaffolds that fill or bridge the lesion, peripheral nerve grafts, or genetic modification to enhance expression of pro-regenerative molecules can also aid in promoting functional recovery.[153–155]

1.2 Breathing Before and After Trauma

Breathing is one of the body's most crucial functions. Injury and disease states that impact breathing are by nature life-threatening and require immediate medical intervention. If the body's natural breathing function cannot be sufficiently maintained, patients may require permanent aids to ensure proper ventilation and adequate tissue oxygenation. Breathing is primarily mediated through rhythmic contractions of the diaphragm muscle, which derives its innervation from motor neuron pools in the cervical spinal cord. The neural circuitry that sustains life by providing excitatory input to these motor neurons can be damaged by high level cervical spinal cord injuries, making the restoration of breathing function a top priority for individuals who sustain an SCI.

1.2.1 Neural Control of Breathing

Breathing is a complex behavior that, by necessity, must be capable of adapting to changes in the internal and external environment. Alterations in oxygen or carbon dioxide levels in the blood, physical exertion, altitude, and many other factors stimulate the body to adjust breathing to ensure that proper tissue oxygenation is maintained in any setting. Multiple respiratory centers in the brainstem exert control over inhalation and exhalation, as well as augmented breathing behaviors that are utilized to clear the airways.

Breathing consists of three phases: inspiration, postinspiration, and expiration. In eupneic breathing, or breathing at rest, breathing rhythm is primarily driven by active muscle contractions of the inspiratory phase, while expiration is typically passive. The rhythmicity is generated by a region of the ventral medulla known as the pre-Bötzinger Complex (preBötC).[156] The groups of neurons from the ventral medulla that would later become known as the preBötC first attracted attention because they maintain discharge patterns consistent with inspiratory rhythm both *in vivo* and *in vitro*, even when isolated from afferent feedback.[156,157] Their axons decussate to communicate with contralateral breathing centers and ensure bilateral synchronicity.[158] Neurons of the preBötC also extend to other pontine or medullary nuclei that modulate ventilatory behaviors including the nucleus tractus solitarius (NTS), the retrotrapezoid nucleus, the nucleus ambiguous, and various regions of the raphe. This densely connected network provides integration of afferent information to adjust breathing in response to an organism's environment.[158,159] Rhythmic firing of excitatory preBötC neurons induces the active inspiratory phase of breathing.[160] Therefore, hyperpolarization or depolarization of these neurons decreases or increases breathing rate, respectively.[161] Death or pharmacologic silencing of pre-BötC

neurons leads to severe breathing deficits and apnea in both rodents and larger species such as goats.[162–164]

Axons projecting caudally from the preBötC communicate with the rostral ventral respiratory group (rVRG) in the caudal medulla. Transmission of the rhythmic signals to the rVRG is crucial as these premotor neurons will ultimately control the pace of diaphragmatic contractions. Premotor neurons in the rVRG provide glutamatergic input to phrenic motor neurons (PMNs) in the ventral horn of spinal cord levels C3-C6.[165–167] PMNs are crucial for inspiration because it is their axons that will exit the spinal cord to form the phrenic nerve. The phrenic nerve innervates the diaphragm and signals it to contract inferiorly, increasing the volume of the thorax and creating negative pressure to draw air into the lungs. Thus, the pre-BötC drives rhythmic inspiration by governing excitatory input to PMNs.[160]

PMNs also receive serotonergic innervation from the caudal raphe, which is crucial for breathing adaptation in response to carotid body activation.[168,169] The carotid body, located at the bifurcation of the common carotid artery, contains chemoreceptors that are sensitive to oxygen levels. Decreases in the arterial partial pressure of oxygen (pO_2) activates the carotid body, which is innervated by the glossopharyngeal nerve. Afferent information from the carotid body is then processed in the nucleus solitarius and the nucleus ambiguous, which communicate with the caudal raphe to modulate serotonergic input to the phrenic motor nucleus.[137,170,171] Neurons of the raphe also possess their own chemoreception capabilities, which is exemplified by their ability to increase the amplitude of phrenic bursting in response to local acidosis.[172] Therefore,

both peripheral and central chemoreception provides a mechanism for modulating breathing activity in response to environmental changes.

Following the inspiratory phase, postinpiration serves to prolong the contraction of the diaphragm, allowing more time for gas exchange across alveolar surfaces.[173] Laryngeal muscles also contract in order to guard against airway collapse during the subsequent expiratory phase.[174] Although the expiration is passive during eupnea, expiratory muscles can be recruited when oxygen levels are reduced. For example, abdominal muscles are recruited during the expiratory phase when rats are exposed to hypercapnic or hypoxic conditions.[175] Expiration also becomes active in response to increased ventilatory demand during exercise and high metabolic rate. In this case, pressure generated by abdominal muscles reduce end-volume of the lungs during expiration, thereby increasing tidal volume.[176] Active expiration is most commonly utilized during speech, laughter, or crying because these expressions require a more rapid inspiratory phase and a more prolonged expiratory phase.[177]

1.2.2 Respiratory Deficits after SCI and Approaches for Restoring Breathing Function

Almost 60% of SCIs occur at the cervical level[2]. Such high level injuries can interrupt the descending pathways that extend from brainstem respiratory nuclei to the C3-C6 spinal cord levels where they synapse on PMNs that innervate the diaphragm. This leads to severe deficits in breathing, often necessitating the use of an artificial ventilator and increasing hospital length of stay and associated financial burdens.[178,179] Ventilator-dependency increases the risk of pneumonia and other respiratory infections, which are the leading cause of death following SCI.[180] Indeed, ventilator use greatly

decreases the life expectancy of injured individuals.[181] Cervical level injury also increases the incidence of sleep disordered breathing, primarily in the form of obstructive sleep apnea, which is a precursor to cardiovascular disease, insulin resistance, and impaired cognitive function.[182–184]

During the first year after injury, many injured individuals who required ventilation are successfully weaned from ventilator use. However, weaning likelihood declines in patients who experience higher level lesions (C1-C3) and in those who undergo tracheostomy, leaving many individuals dependent upon ventilators for extended periods of time.[185] This can lead to ventilator-induced lung injury, as well as diaphragm muscle atrophy.[186–188] In order to restore diaphragm strength and replace the use of a ventilator, some individuals have the option of undergoing implantation of a diaphragm pacer. Diaphragm pacing (DP) requires that electrodes be inserted into multiple sites in the diaphragm in addition to a subcutaneous ground electrode. A pulse generator then electrically stimulates diaphragmatic contractions to maintain breathing while the patient is removed from the ventilator.[189,190] However, if PMNs in the spinal cord or the phrenic nerve itself is injured, this renders DP ineffective.[190] A less invasive approach to stimulating respiratory drive and enhance breathing function after SCI is through therapeutic IH, which will be addressed further in the following section.

1.2.3 Respiratory Motor Plasticity

In the early twentieth century, it was commonly believed that the central nervous system did not change once development was complete. Neuronal cell counts, as well as the synaptic connections these neurons formed were thought to remain unchanged

throughout adult life. However, this dogma was challenged, and ultimately rejected, as evidence emerged revealing the plastic nature of the CNS. One of the first scientists to propose that the CNS changes in response to experience was Donald O. Hebb in 1949.[118,191] As Hebb observed, when a presynaptic cell repeatedly activates a postsynaptic cell, it leads to increased strength of their synaptic connection. In the 1970's, this finding led to the discovery of long term potentiation (LTP), in which stimulation of perforant pathway caused potentiation of excitatory post-synaptic potentials.[192,193] This form of plasticity is dependent upon signaling through NMDA receptors, which then increases trafficking of AMPA receptors to the post-synaptic membrane, thereby strengthening synaptic connections.[194,195]

Since Hebb's time, LTP has become a well-characterized example of plasticity in the brain.[196] Similarly, it has long been known that spinal cord circuits are also capable of changing in response to external stimuli. Like the brain, the spinal cord is capable of instrumental learning, both before and after spinal cord injury.[197–199] In addition, this learning is dependent upon glutamate receptor signaling, which causes synaptic sensitization similar to that produced by hippocampal LTP.[200] While this form of plasticity produces an advantageous increase in synaptic strength, some modes of plasticity in the spinal cord may be maladaptive. For example, following spinal cord injury in rodent models, loss of inhibitory GABAergic signaling, increased sprouting of afferent fibers, and increased membrane trafficking of glutamate receptors all contribute to development of neuropathic pain while impairing spinal learning.[201–205]

Spinal cord plasticity is also evident in the neural control of breathing. In 1895, W.T. Porter discovered that the circuitry responsible for transmitting respiratory signals

from the brainstem to the phrenic motor nucleus exhibited plasticity in response to cervical spinal cord hemisection.[206] This SCI model severs one side of the cord, paralyzing the ipsilateral hemidiaphragm while leaving the contralateral cord intact. Following unilateral interruption of descending respiratory pathways, compensatory breathing activity is exhibited in the contralateral phrenic nerve[207]. In addition, breathing activity returns to the ipsilateral phrenic nerve as respiratory drive intensifies[207,208]. These studies, along with others[6,209,210], demonstrate the potential of spared or latent spinal pathways to modulate breathing activity after cervical SCI. The pathway that Porter detected in the nineteenth century later became known as the crossed phrenic pathway (CPP), which descends from the RVRG and decussates at the level of the phrenic motor nucleus to synapse on contralateral PMN, thereby circumnavigating the cervical hemisection[206,211]. Although it remains latent during eupneic breathing, it can be activated by physiologically or pharmacologically amplifying respiratory drive after SCI, thereby restoring function to the paralyzed hemidiaphagm[207,212]. Presence of the CPP has been validated in a number of species including rats, mice, guinea pigs, dogs, and cats[207,213–215].

Respiratory motor plasticity also manifests as a prolonged increase in breathing motor activity following stimulation of the carotid sinus nerve[216,217] or exposure to intermittent hypoxia[218,219]. This well-described spinal plasticity, termed long term facilitation (LTF), has been shown to augment diaphragmatic (phrenic nerve), external intercostal (intercostal nerves), and upper airway muscle activity (hypoglossal nerve)[220–222]. Depending on the IH protocol used, i.e. length and number of hypoxic bouts and severity of hypoxia, LTF can be induced through either a serotonin- or adenosine-

dependent signaling mechanisms in PMNs[223–225]. These are termed the Q and S pathway, respectively[226]. These signaling cascades and the crosstalk between them have been well described in rats over the past three decades, providing optimized IH protocols and a depth of knowledge for how to modulate the pathways pharmacologically[227–230]. However, very few studies have investigated the impact of IH on the respiratory activity of mice. No standardized IH protocol for mice is currently used across labs. However, hypoglossal and ventilatory LTF have been demonstrated in mouse studies that utilized different levels of hypoxia (7-14%) and number of hypoxic bouts (3-12 bouts, each of a 3-5 minute duration)[231–234].

While exposure to hypoxia stimulates respiratory motor plasticity by increasing respiratory drive, complete elimination of this drive through neural apnea can also lead to inactivity induced facilitation of breathing function[235–237]. Ventilating animals sufficiently to maintain arterial O_2 eliminates respiratory drive. In the absence of respiratory drive, the phrenic nerve ceases firing, causing a neural apnea. A single prolonged period or short repeated bouts of neural apnea lead to augmented breathing activity when respiratory drive returns[235,238]. This form of plasticity has been termed inactivity-induced phrenic motor facilitation (iPMF)[238]. In contrast to LTF, iPMF is induced through either TNF-α or retinoic acid-dependent mechanisms, ultimately activating protein kinase C signaling cascades[236,237,239].

Intrathecal TNF-α dosing is sufficient to induce iPMF, emphasizing the importance of the cytokine's role in plasticity[237]. TNF-α has also been implicated in homeostatic synaptic scaling. Synaptic scaling consists of increases or decreases in excitatory post synaptic potential amplitude caused by activity-dependent changes in the

amount of postsynaptic AMPA receptors[240]. In response to prolonged periods of synaptic inactivity, glia release TNF-α, which stimulates insertion of AMPA receptors into neuronal postsynaptic membranes[34,241]. Thus, TNF-α signaling helps neurons maintain the ability to alter their activity in response to their environment[242]. However, due to the effect of TNF-α on glutamate receptor localization, it also has potential to make neurons more vulnerable to excitotoxic cell death after injury[243]. Systemic inflammation induced by lipopolysaccharide attenuates IH-induced LTF, indicating that inflammatory mediators can have both beneficial and detrimental effects on various forms of plasticity, perhaps dependent on magnitude and duration of inflammatory response[244–246]

1.2.4 Long Term Facilitation of Breathing after SCI

Early studies from Porter and others demonstrated the presence of respiratory drive-induced plasticity in breathing circuitry following spinal hemisection.[206–208] These discoveries prompted investigations into the prospect of alleviating cSCI-induced breathing deficits by increasing respiratory drive through exposure to IH. Almost 20 years ago, Fuller *et al.* showed that IH conditioning could restore ipsilateral hemidiaphragm function following unilateral cervical SCI.[139] The injury model used in this and many other related studies was a lateral C2 hemisection, in which the left half of the spinal cord is transected at the C2 spinal cord level, thereby severing ipsilateral bulbospinal respiratory pathways and rendering the ipsilateral hemidiaphragm chronically paralyzed.[139,218,247] As early as four weeks after injury, therapeutic IH is capable of restoring breathing activity for at least sixty minutes after cessation of IH.[247] Activation of the CPP and manifestation of LTF after injury correlates with the amount of 5-HT

present in the region of the phrenic motor nucleus, which may explain why IH is ineffective at restoring function acutely after injury when 5-HT fiber sprouting has yet to occur.[218,247]

Exposure to IH can also illicit long term facilitation of ventilation in human subjects, both in the presence or absence of cervical spinal cord injury.[142,248] LTF is expressed equally in males and females when subjects are spinally intact.[249] However, data from Tester *et al*. suggests that the ventilatory response to IH may differ between males and females following SCI, providing evidence of enhanced LTF in males.[142] IH is currently in clinical trials to test its impact on a variety of outcomes after SCI, including upper and lower limb function, alleviation of sleep apnea, and breathing (Table 1.).[250–253] In these studies, it will be essential to consider sex as a biological variable to evaluate the true efficacy of IH in the SCI population

1.3 Genetic influences on neural plasticity

Spinal plasticity induced by therapeutic approaches such as exercise and training, electrical stimulation, and intermittent hypoxia have the potential to promote recovery of function after spinal cord injury. However, little is known about how various factors in the human population, such as sex and genetic background of injured individuals, could impact the expression of spinal or respiratory plasticity. This section will discuss how heterogeneity in the human population could modulate the potential for plasticity. Although many of these factors have previously been studied in the context of brain plasticity, they could have additional, unexplored consequences in the spinal cord.

1.3.1 Brain-derived neurotrophic factor

BDNF plays a significant role in modulating dendritic protein synthesis, neurotransmitter release, and glutamate receptor trafficking, demonstrating its importance in regulating synaptic plasticity both pre- and post-synaptically.[254–259] A single nucleotide polymorphism of the *BDNF* gene in which valine is substituted with methionine at codon 66 (Val66Met) has previously been implicated in the inhibition of synaptic plasticity in the brain, resulting in memory impairment.[259–262] In the context of traumatic brain injury, studies in combat veterans demonstrate that BDNF polymorphisms differentially modulate the recovery of cognitive function.[263,264] However, their impact on neural recovery after SCI remains unexplored.

Individuals who express the Val66Met polymorphism have a diminished response to transcranial magnetic stimulation (TMS), which alters the excitability of neural circuits in the brain to improve motor rehabilitation after stroke.[265] TMS has also shown efficacy in improving outcomes after SCI, although it is unknown whether this effect is similarly altered by the Val66Met polymorphism.[266] However, the Val66Met polymorphism does prevent the plastic responses of spinal cord circuitry that are typically induced by electrical stimulation.[267] This SNP could therefore have repercussions for the efficacy of these and other therapies that increase plasticity in the spinal cord to restore function after SCI.[129] For example, spinal plasticity in the form of IH-induced LTF is dependent upon synthesis of new BDNF and its binding to the TrkB (tropomyosin receptor kinase B) receptor.[268] Interestingly, the magnitude of IH-mediated BDNF synthesis has not been compared between wild types and animals expressing Val66Met.

1.3.2 Tropomyosin receptor kinase B

BDNF signals through its receptor, TrkB, to modulate synaptic strength.[258,269,270] The Trk family of receptor tyrosine kinases dimerize upon binding of their neurotrophin ligands, leading to transphosphorylation of the receptor's cytoplasmic domain.[271] Phosphorylated residues then serve as docking sites for signal transduction molecules.[272] Downstream signaling triggered by activation of TrkB increases glutamate-evoked excitatory postsynaptic currents through NMDA receptors.[258,269] Presynaptically, receptor activation increases glutamate release.[273] Mutations in these presynaptic TrkB receptors impair LTP induction, indicating that TrkB signaling modulates synaptic plasticity through multiple mechanisms pre- and post-synaptically.[274] Behavior of TrkB mutant mice suggests that loss of TrkB causes deficits in learning, short term memory, and coping responses to stressful stimuli.[275]

Numerous variants of TrkB are naturally occurring, including splice variants and those containing single nucleotide polymorphisms (SNPs) in the *NTRK2* gene encoding TrkB. Due to the importance of BDNF/TrkB signaling in modulating synaptic plasticity and depressive behavior, a considerable body of work has investigated the impact of these variants on development of depressive disorders in human populations.[276,277] Multiple SNPs within the promotor and intronic regions of *NTRK2* are positively correlated with depression and suicide attempts in human populations.[278–282] In contrast, one TrkB SNP (rs1212171) is correlated with decreased depressive symptoms in females with HIV (human immunodeficiency virus), a population known to experience a higher incidence of depression.[283,284] This disparity points to the complex influence of BDNF/TrkB signaling on synaptic plasticity (previously reviewed by Dwivedi, 2009[285]).

Although it has been established that TrkB signaling is crucial for functional recovery after spinal cord injury[286,287], it is unknown how recovery and response to therapeutics is impacted by polymorphisms in the *NTRK2* gene. Spinal plasticity is dependent upon activation of TrkB, indicating that alterations in TrkB signaling could be detrimental to plastic responses in the spinal cord.[268] As an example of spinally-mediated plasticity, LTF requires BDNF-dependent activation of TrkB receptors.[268] It has been proposed that TrkB then stimulates phosphorylation-induced trafficking of glutamate receptors to the postsynaptic membrane of PMNs, thereby strengthening connections with the CPP.[288] Therefore, variants of the *NTRK2* gene could have either detrimental or beneficial impacts on the induction of LTF, depending on how they alter TrKB ligand binding and kinase activity.

1.3.3 Neurotrophin receptor p75

The p75 neurotrophin receptor binds neurotrophins in both their mature and their uncleaved pro- forms to execute a wide range of functions. During development and after CNS injury, binding of proNGF (nerve growth factor) leads to p75-induced apoptosis.[289] Interaction of p75 and the Nogo receptor inhibits neurite outgrowth in response to myelin-associated proteins.[290,291] The p75 receptor also has a role in regulating hippocampal synaptic plasticity by altering currents through glutamate receptors. Its binding of proBDNF enhances NR2B-mediated currents and increases NMDA receptor-dependent long term depression (LTD).[292] Repeated induction of LTD stimulates production of additional proBDNF, which reduces synaptic strength, as well as dendritic spine density.[293] Knockout of p75 alters levels of glutamate receptor subunits, leading to

impairments in LTD.[294,295] These studies reveal that while mature BDNF strengthens synaptic connections, pro-BDNF depresses synaptic transmission through its interactions with p75.[295]

A polymorphism in the human *p75^{NTR}* gene in which serine 205 is changed to a leucine (S205L) has been associated with decreased incidence of depressive and suicidal behavior compared to individuals with ser205.[296,297] While this could indicate a protective role of this polymorphism in regard to mood disorders, it is unknown whether this polymorphism performs a similar role in other neural disorders or neurotrauma. However, complete inhibition of proNGF/75 receptor interactions improves limb function, coordination, and bladder function after experimental SCI.[298,299] The role of p75 signaling in increasing NR2B-mediated currents is also of not because NR2B-containing NMDA receptors are crucial in mediating hyperexcitability in the dorsal horn, and effect which is associated with neuropathic pain.[300,301] Therefore, the S205L polymorphism could alleviate neuropathic pain if it alters mechanisms of maladaptive plasticity that increase NR2B signaling in the dorsal horn.

1.3.4 Apolipoprotein E

Apolipoprotein E (apoE) is a lipid transport protein that is primarily synthesized in the liver and the brain.[302] In the periphery, apoE is typically a component of very low density lipoproteins (VLDL) and mediates cellular uptake of triglycerides and cholesterol through high-affinity binding to the low-density lipoprotein receptor family. In the CNS, astrocytes are the primary cell type responsible for synthesis of apoE, although microglia and neurons are capable of secreting apoE when injured or stressed.[303–306] ApoE is crucial

for CNS development and plasticity because neurons themselves only produce enough cholesterol for survival. They require apoE-mediated delivery of cholesterol for repair and synapse formation.[307,308] ApoE is encoded by the *APOE* gene, which occurs as three alleles in the human population each differing by single cysteine-arginine substitutions. The alleles are designated as ε2 (cys112, cys158), ε3 (cys112, arg158), and ε4 (arg112, arg158) .[309]

In the 1990's, the ε4 allele gained notoriety as a major risk factor for sporadic Alzheimer's Disease (AD).[310–312] This discovery provided the impetus for numerous studies during subsequent decades that revealed apoE4's association with pathological hallmarks of AD such as amyloid beta plaques and neurofibrillary tangles.[313–315] The ε4 allele, which is expressed in anywhere from 14%-25% of the population, simultaneously increases the risk of developing AD and decreases the age of onset in a dose-dependent manner.[310,316–318] In addition to its role in exacerbating AD pathology, the presence of apoE4 has also been associated with deficits in synaptic plasticity and cognitive function in a variety of other disorders and dementias.[319–322]

Throughout development, synaptic pruning refines the circuitry of the CNS. Some synapses are strengthened, while other are phagocytosed, depending upon their level of activity. Astrocytes play a crucial role in the phagocytosis of unneeded synapses.[323] However, the apoE isoforms differentially modulate this astrocytic function, with E2 enhancing and E4 decreasing phagocytosis and pruning compared to E3-expressing animals.[324] This could explain why apoE4 knock-in mice display deficits in synaptic functioning even at a young age.[325] Deficiencies in synaptic functioning in E4 animals could additionally be attributed to apoE4's modulation of glutamate receptor recycling.

Compared to neurons expressing apoE3 or E2, those expressing human E4 sequester AMPA and NMDA receptors intracellularly. This sequestration is possibly due to an apolipoprotein E receptor 2 (Apoer2)-mediated decrease in receptor phosphorylation.[326,327]

ApoE genotype also influences synaptic integrity through altering the influx of calcium ions. Expression of the apoE4 protein increases influx of extracellular calcium, inducing neurotoxicity *in vitro*.[328] This excessive calcium influx could also be responsible for the increased in calcineurin activity in the cortex of apoE4 targeted replacement mice, which results in a loss of dendritic spines density.[329] In contrast with evidence supporting the presence of excessive intracellular calcium in E4 neurons, a lack of Ca^{2+}-mediated signaling in hippocampi of E4 animals is also evident.[330] Impairment of signal transduction through CaMKIIa (Ca^{2+}/calmodulin-dependent protein kinase IIa) and p-CREB (phosphorylated cAMP response element-binding protein) impairs synaptic plasticity in rats injected with human E4 protein.[330] Together, these results suggest that apoE4-dependent influences on Ca^{2+} signaling may be region-dependent within the CNS, making it difficult to predict how intracellular Ca^{2+} levels may be impacted in the spinal cord.

The detrimental effects of apoE4 on synaptic plasticity have functional consequences. Long term potentiation in the dentate gyrus is attenuated by the E4 isoform.[331,332] Mice expressing human apoE4 have deficits in learning, retention, and spatial memory.[333–335] Deficits in learning and memory are more pronounced in females, as observed in targeted replacement mouse models that express human *APOE* alleles.[336,337] These differences in brain plasticity observed amongst humanized *APOE*

mice are also evident in the human population. Individuals who have at least one E4 allele display deficits in episodic memory and exhibit a decline in cognitive function over time.[338–340]

In addition to its role in neural development and cognitive functioning, apoE is also involved in repair processes following neurotrauma in the CNS and the periphery.[341–343] These processes include neuroprotection, axon regeneration, and synaptogenesis, all of which are impaired in apoE4 mice following CNS insult.[344–346] Indeed, the ε4 allele has been associated with worse outcomes in individuals who experience traumatic brain or spinal cord injuries.[347–349] Studies of traumatic brain injury reveal that apoE4 delays repair of the blood brain barrier, exacerbates ischemic damage, and impairs plasticity in the form of learning and memory.[350–352] Less is known about the impact of *APOE* genotype on plasticity of neural circuits after spinal cord injury, although studies in knockout mice have emphasized that apoE protein is crucial for white matter sparing and functional recovery after SCI.[341,353,354] Data from our lab provides evidence that apoE4 animals exhibit attenuations in spinal and respiratory plasticity both in the absence and presence of cervical spinal cord injury.

1.3.5 Sex as a biological variable in SCI research

A crucial variable that accounts for much of the heterogeneity in the human population but is often not modeled preclinically is sex. Prior to 2009, the majority of preclinical biomedical research was performed in males, with only 15% of studies solely focused on females. Publications that did feature experiments in which both sexes were included did not analyze data in a manner that considered sex a variable, instead grouping

data from males and females.[355] This led to incomplete understanding of disease pathologies and the therapeutic potential of drugs, with women suffering from unforeseen drug-related health risks as a result.[356] In response to this issue as well as the reproducibility crisis, the National Institutes of Health developed policies in 2014 that require grant applicants to include both male and female subjects in their studies unless it can rigorously be proven unwarranted.[357,358]

Studies addressing sex differences in nervous system structure and function have revealed that sex hormones impact CNS inflammation, neuronal dendrite morphology, and synaptic plasticity.[359–362] Sex differences are also evident in the prevalence and manifestation of affective disorders including depression and post-traumatic stress disorder.[363–365] These disparities indicate that the functioning, or malfunctioning, of neural circuits is strongly modulated by sex. Males and females also report different symptoms after traumatic brain injury, with females often experiencing increased anxiety and depression while males display enhanced aggression.[366,367] When compared to males, improved functional outcomes have been observed in females who experience a neurotraumatic even, possibly due to protective effects of estrogen and progesterone.[368–370]

While little is known about the influence of sex on synaptic plasticity after SCI, a series of investigations by Zabka et al. revealed that sex hormones influence 5-HT-dependent plasticity in the spinal cord.[371–373] For example, gonadectomy abolishes LTF in male rats, but can be restored through administration of testosterone, which undergoes conversion to estradiol *in vivo*.[372] Estrogen modulates respiratory motor plasticity by

increasing 5-HT levels in the raphe while progesterone acts as a breathing stimulant.[374–376]

Interestingly, sex effects on CNS plasticity have also been observed in the context of the three *APOE* variants. Brain plasticity in the form of learning and memory is impaired in mice expressing the ε4 allele, an effect that is more severe in females.[336] Until now, the manner in which sex and *APOE* genotype interact to influence plasticity and recovery of function after SCI has remained uninvestigated. However, conclusions from previous studies collectively emphasize the importance of considering both sex and genetic background when developing treatment strategies for the diverse spinal cord injured population since individuals may be predisposed toward or against plasticity and functional recovery. Indeed, a recent review from Fouad et al. emphasized the importance of considering variation in the human population and the response to therapeutics as a key to improving translation.[377] Current work in our lab has aimed to address this gap in knowledge and provides novel insight into the interplay of sex and the human *APOE* alleles that modulates spinally-mediated plasticity in the presence or absence of injury.

CHAPTER 2. HUMAN APOE AS A BARRIER TO RESPIRATORY MOTOR PLASTICITY IN RATS

2.1 Introduction

The majority of spinal cord injuries (SCIs) occur at the cervical level. [2] These high-level injuries can disrupt axons that descend from the brainstem to synapse on phrenic motor neurons (PMNs) at the C3-C6 level. PMNs control the contraction of the diaphragm, which is the primary muscle responsible for breathing function, and damage to these bulbospinal pathways leads to significant breathing impairments. As a result, cervically injured individuals who do not achieve sufficient spontaneous recovery become reliant on artificial ventilation, increasing their risk for respiratory infections and death after injury. Indeed, the restoration of independent breathing function is a top priority for the SCI community.[378]

Therapeutic approaches that are being developed preclinically with the goal of improving breathing function after SCI are plentiful and diverse, yet clinically available therapeutics are extremely limited. Currently, the primary commercially available method of restoring diaphragmatic function is surgical implantation of diaphragm pacers.[190] Less invasive approaches to improve motor function, such as transcranial magnetic stimulation and intermittent hypoxia (IH) often work through a mechanism of modulating plasticity in the spinal cord to induce lasting changes in neural circuitry function.[266,288,379] However, preclinical investigations into the efficacy of these strategies often utilize homogenous populations of animal models that are incapable of reflecting the diversity found in the human population. Therefore, the question of how plasticity in the spinal cord may be modulated by genetic factors currently remains unanswered. Indeed, human genetic

diversity could constitute a major confounding factor in clinical studies. For example, when therapeutics which show preclinical efficacy are advanced into clinical trials only to show conflicting or variable results. Ultimately, genetic variation could contribute to the lack of bench to bedside translation for the SCI population.

One well-described form of plasticity that has been validated to restore function to the paralyzed diaphragm after cervical SCI is long term facilitation (LTF). LTF can be induced through exposure to intermittent hypoxia (IH), which activates BDNF (brain-derived neurotrophic factor)- and glutamate receptor-dependent synaptic strengthening.[268,380] While LTF was first described as a prolonged augmentation of respiratory activity in intact animals, it was later discovered that it is also sufficient to improve diaphragm function in spinally injured animals.[139,224,247] In addition to respiratory LTF, IH also induces plasticity in other motor systems. Indeed, IH has been utilized in clinical trials as a therapeutic approach to improve hindlimb, forelimb, and ventilatory outcome in SCI populations.[140–142] Despite consistent preclinical findings, clinical studies have demonstrated that up to one-third of patients are "non-responders" to IH, meaning that they show no benefit from treatment.[141,381] This indicates that factors in the human population, which are not represented in preclinical animal models, may limit the propensity for plasticity in some individuals, thereby reducing the potency of IH as a therapeutic tool.

The vast majority of preclinical investigations of the respiratory response to IH have been performed in rats. This has advantageously allowed for the optimization of IH protocols and provided a thorough understanding of the molecular mechanisms behind LTF in this particular animal. For example, because LTF is known to be serotonin (5-

HT)-dependent, MacFarlane and Mitchell (2009) demonstrated that LTF can also be induced through episodic 5-HT dosing of the spinal cord. Unfortunately, the dependence on rat models of LTF limits the potential for studying the influence of human genetic diversity on this plasticity. To circumvent this deficiency while still utilizing a well-characterized rat model of respiratory motor plasticity, the current study applies exogenous human apolipoprotein E (apoE) proteins directly to the spinal cord prior to episodic 5-HT dosing to determine how different isoforms of a human protein could alter spinal plasticity both in the presence and absence of cervical SCI.

Of the three isoforms of apoE, designated E2, E3, and E4, the E4 isoform has previously been associated with impaired synaptic plasticity. The E3 and E4 isoforms (encoded by the ε3 and ε4 alleles of the *APOE* gene) are much more prevalent than E2, which is only present in around 7% of the human population.[318] Therefore, the E3 and E4 isoforms of apoE are ideal targets to test whether genetic factors in the human population may limit individuals' propensity for plasticity, and consequently reduce the efficacy of therapeutic approaches that harness spinally-mediated plasticity as a means of improving functional recovery after SCI. We hypothesize that rats dosed with human apoE4 will display an abatement in respiratory motor plasticity, emphasizing the importance of considering human genetic diversity when developing and translating SCI therapeutics.

2.2 Materials and methods

2.2.1 C2 Hemisections

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Female Sprague-Dawley retired breeder rats

were obtained from Envigo (Indianapolis, IN). Animals were acclimated for at least one week before injury C2 hemisection. Prior to surgery, rats were anesthetized with ketamine (75-100mg/kg, Ketaset, Zoetis) and xylazine (5-10 mg/kg, AnaSed, Akorn Pharmaceuticals). When the proper plane of anesthesia was reached, the surgical site was shaved and sterilized with alternating 70% ethanol and betadine swabs. Puralube ophthalmic ointment was applied to the eyes to prevent drying during surgery. A skin incision was made on midline, extending from the ears to the level of the scapula. An incision was then made through the acromiotrapezius, semispinalis capitus, and rectus capitus muscles along the midline and muscles were retracted. Paravertebral musculature wass cut away from the C2 vertebra. A C2 laminectomy was performed by cutting the pedicles of the C2 vertebra bilaterally. The dura was perforated and resected using microscissors. The tip of a 27 gauge needle was bent at a 45 degree angle. Under a dissecting microscope, the needle tip was then inserted at the midline of the spinal cord at the C2 level, caudal to the C2 dorsal roots, and a cut was made extending from midline to the lateral edge of the cord. The needle was passed through the spinal cord in the same location for a total of three passes to ensure lesion completeness. Musculature was sutured with 6-0 absorbable suture and skin was closed with wound clips. Rats received subcutaneous buprenorphine (0.05mg/kg) and carprofen (5mg/kg) immediately as they began regaining consciousness.

2.2.2 Chronic Spinal Cord Exposures and Episodic Serotonin Dosing

Episodic 5-HT dosing was performed 20 weeks post-injury in C2 hemisected animals. Episodic 5-HT dosing utilized the same protocol for spinally intact and C2

hemisected rats. Animals were anesthetized with urethane (1200mg/kg) intraperitoneally. An incision was made through the skin on the dorsal aspect of the neck as previously described for a C2 hemisection. Musculature and scar tissue were removed to expose the spinal cord at the C2/C3 level and a durotomy was performed with microscissors. Once the spinal cord was exposed, animals were moved into a supine position and a laparotomy was performed by incising the rectus abdominis, external oblique, and internal oblique muscles. Bipolar electrodes, connected to an amplifier and data acquisition system (CED 1401 with Spike2 Data Analysis Computer Interface, Cambridge Electronic Design), were inserted into the left and right hemidiaphragm and ground electrodes were inserted into the hind paws. Abdominal muscles were sutured and the animal was returned to prone position.

With animals in prone position, 20ul of apoE protein (1μg/ml in physiological saline, optimal concentration was determined by preliminary studies, data not shown) was applied to the spinal cord. After 30 minutes, the apoE solution was swabbed from the spinal cord and 6ul of 5-HT (1μM in apoE solution, as described by MacFarlane and Mitchell, 2009) was applied to the spinal cord. After 5 minutes, the first dose of 5-HT was absorbed with a swab and the second dose was applied. This was repeated for a total of three doses (Figure 1 *B*). Diaphragmatic activity was recorded for 40 minutes after the final 5H-T dose. Animals were then perfused with 4% paraformaldehyde (PFA) and the spinal column was removed and placed in 4% PFA at 4°C. After 2 days, spinal columns were removed from PFA and placed in 30% sucrose at 4°C for cryoprotection until sectioning.

2.2.3 Diaphragmatic EMG trace analysis

After recording, raw diaphragmatic EMG traces were rectified and integrated using Spike2 software. Analysis was performed at six time points: twice during baseline recording, once 5 minutes before the final 5-HT dose, and at 5, 20, and 40 minutes after the final 5-HT dose (Figure 2.1 *B*). For each time point, peak amplitude was averaged over a 30 second period. Amplitude of diaphragmatic bursts at each time point were normalized to that animal's pre-hypoxia baseline amplitude. Baseline amplitude was calculated by averaging the amplitudes quantified at 10 minutes and 5 minutes before the first dose of 5-HT.

2.2.4 Labeling of phrenic motor neurons

Labeling of PMNs was performed as previously described by Mantilla et al. [383] Briefly, three days prior to terminal EMG recordings, animals received bilateral intrapleural injections of recombinant cholera toxin subunit B (CTB) conjugated to AlexaFlour 488 (Invitrogen, C22481). CTB was diluted in physiological saline to a concentration of 0.2%. Animals were anesthetized with isoflurane. The ventral thoracic region was shaved and swabbed with 70% ethanol. Using a Hamilton syringe, 20μl of diluted CTB was injected in each the left and right sides of the thoracic cavity at a depth of 6mm from the skin.

2.2.5 Sectioning and Immunohistochemistry

Spinal cords were dissected out of the spinal columns and embedded in Optimal Cutting Temperature embedding medium (OCT, Sakura Tissue-Tek). Tissue was

cryosectioned at a thickness of 30μm and placed directly onto slides. For the injury site (C2), every other section was collected. For the region of the phrenic motor nucleus (C3-C6) every section was collected serially onto 10 slides so that every slide held sections from each spinal cord level. Sections from the C3-C6 were then labeled with antibodies for synaptophysin (Invitrogen 50-6525-82, diluted 1:32) and either GluR1 (Abcam Ab31232, diluted 1:500) or NR1 (Abcam 52177, diluted 1:100). Antibodies were diluted in blocking buffer (5% normal goat serum, 0.1% bovine serum albumin, and 0.1% triton-X100 in 1xPBS). Slides were mounted with Flouromount (Southern Biotech, cat No. 0100-20) and immediately sealed with clear nail polish.

2.2.6 Imaging and Colocalization Analysis

Imaging was performed on a Nikon Eclipse T*i* series inverted confocal microscope at 20x. For staining quantification in tissue from uninjured animals, snapshots of phrenic motor neurons were captured at the C4 level of each animal. For quantification in injured animals, Z stacks (2.19μm per step) of phrenic motor nucleus were taken at the C4 level both ipsilaterally and contralaterally to injury. Investigators were blinded to genotype during imaging and quantification.

For colocalization analysis, images were opened in ImageJ (National Institutes of Health). Investigators were blinded to treatment and injury status until after quantification was complete. A region of interest was drawn around each phrenic motor neuron labeled by CTB. Colocalization was quantified through the “colocalization threshold” plugin. The Costes method for statistical significance was used to calculate Pearson’s correlation coefficient between randomized images and a study image to ensure that detected

colocalization was real (ImageJ, “Colocalization Analysis”, 2020). To determine the percentage of synaptic glutamate receptor subunits compared to total subunits in each PMN, the number of colocalized voxels was then normalized to all voxels that were positive for NR1 or GluR1 in the region of interest.

2.2.7 Quantitative PCR

Fresh spinal cord tissue was collected from a cohort of animals for mRNA and protein quantification. These animals were sacrificed 1 hour after the final dose of 5-HT and the region of the phrenic motor nucleus (C3-C6) was excised and immediately frozen on dry ice. Tissue was kept at -80°C until analysis was performed. The tissue was then divided for protein or RNA isolation, with 35-40mg of tissue being used for RNA extraction. Tissue was homogenized using a Bead Ruptor (Omni International). RNA was purified using an RNeasy Lipid Tissue Mini Kit (Qiagen, Cat #74804) and quantified by NanoDrop (ThermoFisher Scientific). Reverse transcription was performed in a Thermal Cycler (Bio-Rad) using 25ng/µl of sample RNA. Quantitative PCR was performed on 25ng of cDNA in a QuantStudio 7 (Applied Biosystems) using Luna Universal Probe Master Mix (New England BioLabs, Cat #M3004S) and primers for BDNF (ThermoFisher, Rn01484924_m1) and actin (ThermoFisher, Rn00667869_m1). Fold change from vehicle-treated animals was then calculated for E3- and E4-treated animals.

2.2.8 BDNF Quantification

Remaining fresh tissue was homogenized in RIPA buffer (Alfa Aesar, J62524) with protease inhibitor (Pierce, Cat #A32965) for protein isolation. Protein was

quantified by a BCA assay (Pierce, Cat #23227). Protein was quantified using a Meso Scale Discovery UPLEX Assay (C0292-2).

2.2.9 Statistical Analysis

For statistical analysis of EMG traces, repeated measures (RM)-ANOVA was used to account for within-subject correlation given repeated measurements over time. Additionally, Student's t-tests were performed at three time points (5 minutes before final 5-HT, 5 minutes after final 5-HT, and 40 minutes after 5-HT) to determine whether there was a significant difference in diaphragmatic burst amplitude at any single time point. However, RMANOVA was the primary statistical analysis for this data. One injured apoE3-dosed rat was excluded due to its classification as a significant statistical outlier according to the Grubbs test. Two-way ANOVAs were used to analyze colocalization staining. Student's t-tests were used to analyze qPCR and ELISA data. Results were considered statistically significant if $p < 0.05$. Investigators were blinded until all diaphragmatic EMG and histology analyses were complete.

2.3 Results

2.3.1 Respiratory motor plasticity in the presence of human apoE isoforms

Episodic 5-HT dosing of the cervical spinal cord is sufficient to induce LTF, which manifests as prolonged augmentation of breathing motor output.[382] Utilization this method of induction, in contrast to exposure to IH using hypoxia chambers, allowed for simultaneous recordings of diaphragmatic EMGs. Human apoE protein in either the E3 or E4 isoform was applied intrathecally (1μg/ml) to the exposed spinal cord at the C3-C6 level, where phrenic motor neurons are located (Fig. 2.1 *A*). After thirty minutes of

baseline diaphragmatic EMG recording in the presence of human apoE, rats received three doses of 5-HT (1μM) separated by 5 minutes (Fig. 2.1 *B*). Diaphragmatic activity was recorded for 40 minutes after the final dose of 5-HT. Representative traces from E3 and E4-treated animals are shown in Fig. 2.1 *C.* Quantification of burst amplitude demonstrated that there was no difference in the 5-HT-induced breathing changes between animals exposed to E3 and E4 protein (Fig 2.1 *D,E*). Time points for burst amplitude quantification are indicated by green arrows in Fig. 2.1 *B.*

2.3.2 The influence of apoE isoforms on synaptic glutamate receptor levels

LTF is dependent upon activation of glutamate receptors on phrenic motor neurons.[384,385] Because apoE4 is known to impair glutamate receptor trafficking[326], we quantified the amount of synaptic AMPA and NMDA receptors on CTB-labeled phrenic, as previously described by Huie et al. (2015)[203], motor neurons to determine whether E4 would impede synaptic localization. Fig. 2.2 *A-B* shows representative images of colocalized staining of the synaptic marker synaptophysin and either the NMDA receptor subunit NR1 (*A*) or the AMPA receptor subunit GluR1 (*B*) on PMNs. Synaptic glutamate receptor subunits were normalized to the total amount of intracellular subunit staining. Quantification of synaptic NR1 and GluR1 demonstrated no significant difference between genotypes (Fig. 2.2 *C-D*, 2-way ANOVA, NR1 $p=0.21$ and GluR1 $p=0.31$). However, a trend emerged in which episodic 5-HT led to increases in synaptic localization of NR1 in rats exposed to E3 and E4 (Fig. 2.2 *C*). While a similar trend of 5-HT-induced upregulation was observed in synaptic GluR1 in E3-treated animals, it was not evident in animals receiving E4 protein (Fig. 2.2 *D*). An inability to increase synaptic

glutamate receptors in response to 5-HT suggests a potential apoE4-dependent deficiency in the ability to initiate synaptic plasticity.

2.3.3 ApoE isoform-dependent modulation of spinal plasticity after SCI

In addition to enhancing breathing activity in intact rats, LTF also restores breathing function to the paralyzed hemidiaphragm after cervical SCI.[139,247] To determine whether the impact of human apoE isoforms on spinally-mediated respiratory motor plasticity are altered by SCI, we performed episodic 5-HT dosing on C2 hemisected rats. In order to increase clinical relevance, we performed this 5-HT dosing protocol on animals that were chronically injured (20 weeks) when they received episodic 5-HT concurrently with terminal diaphragmatic EMG recordings. All rats in this study displayed spontaneous recovery of diaphragmatic recovery by 20 weeks post injury (data not shown). Fig. 2.3 *A* illustrates the lateral C2 hemisection injury model and depicts the resultant interruption of bulbospinal respiratory axons. As depicted in Fig. 2.3 *B*, our injury completely destroys the left half of the spinal cord at the C2 level. Diaphragmatic EMG recordings were performed concurrently with human apoE isoform administration and subsequent episodic 5-HT dosing as in intact animals. In contrast to intact animals, injured animals receiving apoE3 appeared to increase breathing activity after the final dose of 5-HT (Fig. 2.3 *C*). This is analogous to the effect observed in rats dosed with episodic 5-HT in the absence of human apoE.[382] Although injured apoE4-treated animals did not exhibit a similar augmentation in breathing, there was no statistically significant difference between groups (Fig. 2.3 *D-E*, RM ANOVA p=0.58). Diaphragmatic burst amplitude peaked at 5 minutes after the final dose of 5-HT in E3 animals. However, there

was no significant difference between amplitude in E3 and E4 animals at this time point (Fig. 2.3 *E*, Student's t-test p=0.27).

2.3.4 Synaptic glutamate receptor expression on phrenic motor neurons after SCI

Similar to the procedure performed on tissue from spinally intact rats, tissue harvested from C2 hemisected rats was stained for synaptophysin and either NR1 (Fig. 2.4 *A*) or GluR1 (Fig. 2.4 *B*). Colocalization of these molecules was quantified on PMN that had been retrogradely labeled with CTB, then normalized to total glutamate receptor subunits in the cell. Quantification of NR1 colocalization revealed that there was no difference between E3 animals that did receive episodic 5-HT and those that didn't (2-way ANOVA, multiple comparisons with Tukey's post hoc test p=0.97). In contrast, 5-HT induced a significant reduction in synaptic NR1 levels in E4-treated animals (Fig. 2.4 *C,* 2-way ANOVA, multiple comparisons with Tukey's post hoc test p=0.0266). The pattern of synaptic localization of GluR1 in injured animals differed greatly from that of NR1. Episodic 5-HT dosing appears to upregulate the synaptic localization of GluR1-containing AMPA receptors in E3 animals, although this trend did not reach statistical significance (Fig. 2.4 *D,* 2-way ANOVA, multiple comparisons with Tukey's post hoc test p=0.075). Episodic 5-HT treatment did not lead to changes in synaptic GluR1 levels in animals with apoE4 (2-way ANOVA, multiple comparisons with Tukey's post hoc test p=0.60). This supports the trend observed in spinally intact E4 animals, further insinuating that E4 prevents 5-HT-induced increases in GluR1 trafficking to the postsynaptic membrane of PMNs.

To determine whether C2 hemisection alone stimulated changes in synaptic glutamate receptor levels, we also compared NR1 and GluR1 colocalization between intact and injured animals that did not receive episodic 5-HT dosing. Figure 2.4 *E-H* shows this quantification in E3 (*E,G*) and E4 (*F,H*) animals. In both E3 (*E.* Student's t-test $p<0.0001$) and E4 (*F.* Student's t-test $p<0.0001$) treated animals, synaptic NR1 colocalization significantly decreases after injury. However, synaptic GluR1 localization did not differ significantly between uninjured and injured animals treated with human E3 (*G.* Student's t-test $p=0.29$) or E4 (*H.* Student's t-test $p=0.41$) protein.

2.3.5 Influence of apoE isoforms on 5-HT-dependent BDNF production

Previous studies have shown that induction of LTF leads to production of new BDNF following 5-HT receptor activation.[223,268] Since this BDNF synthesis and subsequent binding of its TrkB (tropomyosin receptor kinase B) receptor is necessary for LTF, we investigated whether BDNF production differs between E3- and E4-treated animals. In uninjured animals, presence of human apoE3 or apoE4 did not appear to influence BDNF synthesis at either the mRNA or protein level in the region of the phrenic motor nucleus (Fig. 2.5 *A,C,* Student's t-tests, mRNA $p=0.73$, protein $p=0.69$). Likewise, mRNA and protein quantification in tissue harvested from injured animals exposed to episodic IH revealed no significant difference between animals treated with E3 and E4 (Fig. 2.5 *B, D,* Student's t-tests, mRNA $p=0.83$, protein $p=0.43$). Comparison of vehicle-treated animals that did not receive human apoE protein prior to episodic 5-HT dosing demonstrated no significant difference between BDNF mRNA expression in intact and injured animals (Fig. 2.6, Student's t-test $p=0.74$). This suggests that although

episodic 5-HT did not induce the expected increase in BDNF levels in hemisected animals, this was not due to an injury-induced ceiling effect on BDNF production.

2.4 Discussion

Harnessing neural plasticity in the spinal cord by strengthening synaptic connections or activating spared latent pathways can provide a means of improving functional recovery after spinal cord injury. While therapeutic approaches that enhance neuroplasticity have proven effective in homogenous populations of preclinical rodent models, the impact of genetic variation in the diverse human population remains underexplored. The current study represents the first investigation into how genetic variation may impact individuals' predisposition towards neuroplasticity, and consequently, their response to treatment strategies after SCI. By utilizing a well-described model of respiratory motor plasticity in rats, we were able to model the impact of the plasticity-limiting human apoE4 protein on serotonin-dependent recovery of breathing function after SCI. Contrary to our hypothesis, we did not observe significant differences in the respiratory response to IH between E3 and E4 treated animals. However, apoE4 did cause a decline in synaptic NMDA receptor levels, indicating that apoE isoforms modulate plasticity at the molecular level in PMN synapses or in intracellular signaling cascades that are activated by therapeutic IH or episodic 5-HT.

2.4.1 Human apoE isoforms and plasticity in the neural control of breathing

Long term facilitation of respiratory activity was first discovered as a result of carotid sinus nerve or carotid body afferent stimulation.[386] This plasticity can also be induced through hypoxic stimulation of the carotid body, but only if the hypoxia

exposure consists of repeated bouts opposed to a single, sustained hypoxic period.[387] Both electrical and hypoxic stimulation of carotid body afferents induce respiratory motor plasticity through serotonin-dependent mechanisms that result in strengthening of synaptic inputs onto PMNs.[217,224,388] Indeed, directly administering episodic doses of 5-HT directly to the cervical spinal cord of rats activates LTF through a mechanism similar to that of IH.[382] However, episodic 5-HT in animals that had human apoE3 or E4 protein present in the cervical spinal cord did not induce augmentation of breathing motor output that is typically characteristic of LTF.

The lack of LTF observed in apoE-treated rats could be due to a variety of factors. The original studies that describe LTF of breathing utilized animals that were vagotomized and artificially ventilated to maintain phrenic activity at the level that would be present during eupneic breathing. This was accomplished through monitoring pCO_2, which created a feedback loop with the ventilator to preserve eupneic activity levels. Exhaled CO_2 and body temperature were also monitored to control for changes in metabolic rate, cardiac output, and resulting CO_2 return to the lungs.[386,389] While rats in the present study were kept on heating pads throughout IH and diaphragmatic EMG recordings, body temperature and CO2 levels were not monitored. As a result, animals may have experiences hypoxia-induced metabolic adjustments or vagal feedback that led to changes in breathing, which could negate the induction of LTF.

Apolipoprotein E is a prominent protein of interest in the Alzheimer's Disease (AD) literature, due to its designation as a significant risk factor for developing AD in people who express the apoE4 isoform. As a result, thousands of publication have described its role in the central nervous system in health and disease, although very few

studies have examined its role in spinal cord repair and recovery of function after SCI. Interestingly, spinally injured individuals who are apoE4+ exhibit less motor recovery than those who don't carry the allele encoding E4.[348,390] Although mouse studies have revealed that apoE levels in the spinal cord are elevated for up to three weeks post-injury, it is unknown how the distinct human isoforms may influence recovery or the response to therapeutics such as IH after SCI.[341,391] While our data indicates that there is no difference in the response to episodic 5-HT between E3 and E4-treated animals, there are some methodological considerations to be addressed. The cervical spinal cord was bathed in human apoE protein for 30 minutes prior to episodic 5-HT. However, it is unknown whether apoE penetrated the cord sufficiently to reach PMN in the ventral horn. Additionally, apoE could have been interacting with various cell types in the spinal cord including astrocytes, microglia, and interneurons to influence signaling in the spinal circuitry. Rats were also still producing the rat form of apoE, which may have been competing with the exogenous human isoforms for receptor binding. These uncontrolled circumstances could have dampened the effect of the human isoforms in the spinal cord.

The human apoE proteins used in these experiments were unlipidated. ApoE functions as a lipid carrier in the CNS and is lipidated by the ABCA1 (ATP binding cassette A1) transporter. ABCA1 transports cholesterol and phospholipids into HDL-like molecules that contain apoE.[392] Lipidated apoE then binds its receptors such as LDLR (low density lipoprotein receptor) or apoer2.[393,394] Because the different isoforms of apoE are known to have different lipidation profiles and also display isoform-dependent interactions with apoE receptors, the lack of lipidation may have altered or inhibited the capacity of human apoE isoforms to enter cells through receptor binding in the rat

CNS.[395–397] To address these methodological concerns, we have performed additional studies in targeted replacement *APOE* mice, which express the human *APOE* alleles instead of the murine form under the control of the mouse promoter, allowing the animals to produce physiologically relevant lipidated human apoE protein for the duration of their lives, including during the recovery period following SCI.[398–400]

2.4.2 The influence of human apoE on plasticity in the injured cord

The restoration of breathing function after SCI is crucial to limit hospital costs and lengths of stay, increase life expectancy, and decrease the occurrence of comorbidities such as respiratory infections.[179,401,402] Intermittent hypoxia is therefore an intriguing therapeutic approach because it not only enhances spinally-mediated plasticity for the improvement of respiratory motor function, it also modulates plasticity in other motor systems. IH has undergone clinical trials with positive outcomes for ventilation, hand function, and ankle strength.[140,142,403,404] However, subjects demonstrate varied responses to IH treatment, with some demonstrating notable functional improvement while others appear unaffected or even exhibit a slight decline in function.[141,381] While the heterogeneous nature of SCIs could account for some of this variability, genetic variability that isn't represented in preclinical animal models could also interfere with individuals' capacity to respond to therapeutic approaches that stimulate plasticity of spinal circuits.

In support of this hypothesis is the emergent trend in C2 hemisected animals that were exposed to human apoE isoforms. While those treated with E3 displayed the expected increase in diaphragmatic burst amplitude following episodic 5-HT, E4 animals

had no change in breathing activity. Although this trend did not reach significance, it indicates that the human apoE isoforms may have a role in modulating respiratory motor plasticity. ApoE4 has gained notoriety for impairing synaptic plasticity in the brain, but this is the first investigation to find similar effects in spinally-mediated forms of plasticity.[331,405] The dosing strategy utilized in this study had been previously described in rats by MacFarlane and Mitchell (2009). However, since both human apoE protein and 5-HT were applied as solutions to the dorsal surface of the exposed spinal cord, there could have been inter-animal variation in the diffusion patterns of these molecules, explaining the variation observed between animals that prevented our data from reaching statistical significance. Future studies utilizing targeted replacement *APOE* mice will nullify this concern by ensuring that human apoE is expressed equally under the same promoter in all animals.

2.4.3 Synaptic glutamate receptor levels on PMN after exposure to human apoE

Although statistically significant differences in the diaphragmatic activity of E3 and E4 animals were not observed, this does not rule out the possibility that the human apoE isoforms influences synaptic plasticity through a mechanism that isn't robust enough to be observed in EMG recordings. LTF is induced through a signaling cascade in PMN that relies on newly synthesized BDNF signaling through the TrkB receptor, ultimately leading to phosphorylation and trafficking of glutamate receptors to the postsynaptic membrane.[268,288] These postsynaptic glutamate receptors are crucial for synaptic strengthening of synaptic inputs on PMN and are necessary for the induction and maintenance of LTF.[384,385] A lack of synaptic glutamate receptors on PMN would

therefore indicate a deficiency in the ability to induce spinal plasticity. ApoE4 is known to decrease trafficking of both AMPA and NMDA receptors, leading to deficits in synaptic plasticity.[326] By utilizing a colocalization staining method described by Huie et al. (2015), we found that uninjured animals displayed no difference in synaptic AMPA or NMDA receptor subunit levels, regardless of apoE isoform or episodic 5-HT exposure. However, our data did indicate that 5-HT dosing led to slight increases in NR1 synaptic localization in both E3 and E4 animals, but only increased GluR1 in those with E3. Interestingly, although E4 animals did not demonstrate a similar increase in GluR1 after episodic 5-HT, they did have higher levels of GluR1 in the absence of 5-HT. Thus, the absence of GluR1 upregulation could indicate a ceiling effect on synaptic AMPA receptors, perhaps to compensate for the reduced dendritic spine density and loss of synaptic integrity that has been associated with apoE4.[329,407]

Glutamate receptor localization after C2 hemisection differed greatly from that observed in intact animals. Both E3- and E4-treated animals downregulated synaptic localization of NR1 after injury. Perhaps more striking was the finding that episodic 5-HT dosing caused a significant decrease in synaptic NMDA receptors when apoE4 was present. Since LTF requires signaling through NMDA, but not AMPA, receptors, this loss of postsynaptic NMDA receptors could contribute to the effect of E4's dampening effect on the respiratory response to IH in injured animals. NMDA receptors (NMDAR) are heterotetramers composed of NR1, NR2, and NR3 subunits.[408] While NR1 is an obligate subunit of functional NMDA receptors and therefore indicative of total NMDARs present, future investigations into the synaptic presence of additional NMDAR subunits would provide greater insight into how synaptic plasticity is altered by human

apoE isoforms. For example, previous work has shown that synaptic strengthening in the form of cortical long term potentiation relies on NR2A-containing NMDAR.[409] Similarly, spinal cord plasticity leading to spontaneous recovery of breathing function and activation of the crossed phrenic pathway (the spared anatomical substrate mediating LTF after C2 hemisection) correlates with NR2A levels on phrenic motor neurons.[410,411]

Subunits of AMPA receptors, GluR1 , 2, 3, or 4, also have unique impacts on synaptic signaling and plasticity, particularly since the GluR2 subunit confers calcium impermeability.[412] Decreases in GluR2 after SCI are associated with synaptic strengthening mediated by calcium-permeable AMPA receptors.[411,413] However, respiratory drive to PMN is communicated through glutamatergic bulbospinal pathways, emphasizing the importance of synaptic AMPAR expression.[414,415] Accordingly, in response to SCI, GluR2 subunits decline as levels of the GluR1 subunit rise, although the increase in GluR1 after SCI in our apoE-treated animals did not reach statistical significance.[411] Due to the different signaling properties conferred by various NMDA and AMPA receptor subunits and their role in modulating plasticity, further studies are needed to determine how additional subunits are altered in E3- and E4-treated animals.

2.4.4 BDNF synthesis in response to episodic 5-HT

The differences observed in synaptic glutamate receptor localization following exposure to episodic 5-HT dosing between animals whose spinal cords had received apoE3 or E4 suggest that there could be apoE isoform-dependent influences on 5-HT-induced signaling mechanisms upstream of glutamate receptor trafficking. Synthesis of new BDNF is necessary for downstream glutamate receptor trafficking and the induction

of LTF.[268] ApoE4 is associated with lower levels of BDNF in the brains of targeted replacement mice, making this step in the signaling cascade a likely candidate for apoE4's inhibitory effect on synaptic glutamate receptor localization, and subsequent dampening of respiratory motor plasticity.[416] Contrary to expectation, levels of BDNF mRNA and protein did not differ between spinal cord tissue from E3- and E4-treated rats. BDNF initiates downstream signaling through binding of its TrkB receptor.[268] Although some evidence indicates that TrkB expression and kinase activity are attenuated by apoE4, other studies have suggested otherwise.[416,417] Further investigation into the expression and activity of TrkB in the spinal cord of E3 and E4 animals may contribute to the understanding of the mechanism leading to loss of synaptic glutamate receptors that is associated with E4.

Through measuring diaphragmatic EMG recording and histological analysis, we show that the variant human isoforms of the apoE protein modulate plasticity in spinal circuits. Considering that many therapeutic approaches for SCI are developed to promote functional recovery by enhancing spinal plasticity, this data has important implications for how genetic diversity in the SCI population may influence individuals' response to treatment strategies. Indeed, previously understudied factors such as *APOE* genotype could contribute to the lack of translation encountered by many SCI therapeutics.

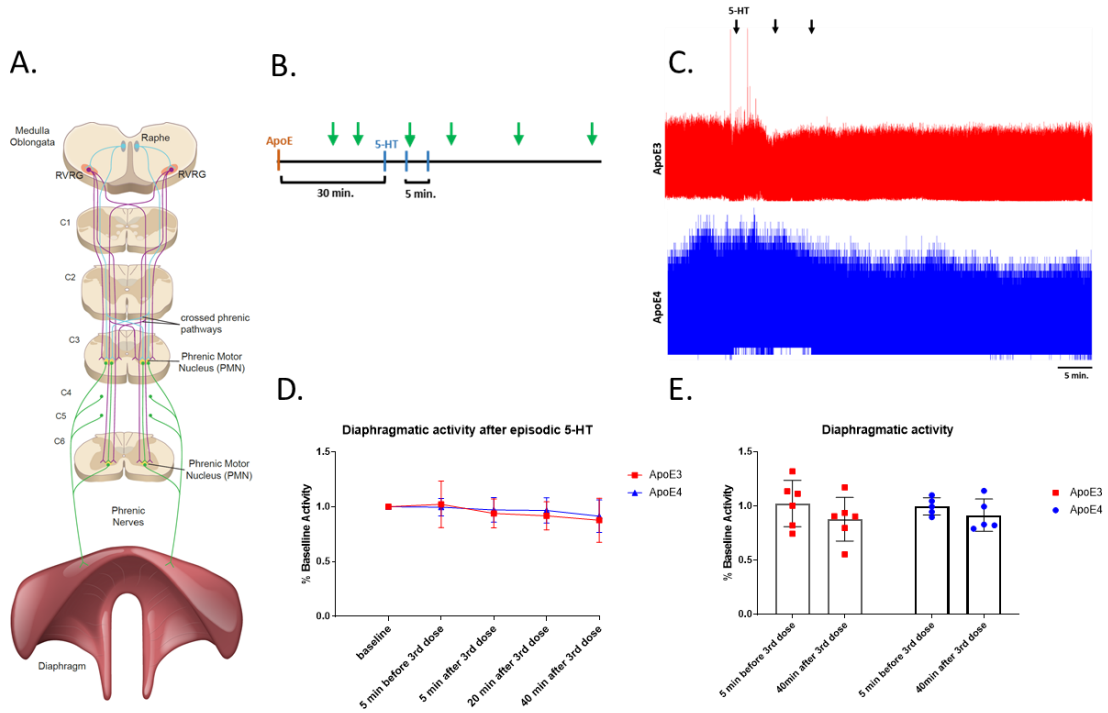

Figure 2.1 Human apoE3 and E4 affect 5-HT dependent plasticity similarly.

A. Schematic of the neural circuitry that mediates breathing. *B.* Timeline of the episodic 5-HT dosing protocol with concurrent diaphragmatic EMG recordings. *C.* Representative diaphragmatic EMG traces illustrating breathing activity during and after episodic 5-HT. *C.* Quantification of diaphragmatic burst amplitude over time in response to episodic 5-HT. There was no significant difference between animals exposed to E3 and E4 (RM ANOVA p=0.762). *E.* Quantification of diaphragmatic amplitude 5 minutes before and 40 minutes after the final dose of 5-HT (Student's t-tests E3xE4 5min before final dose p=0.80, E3xE4 40min p=0.74)

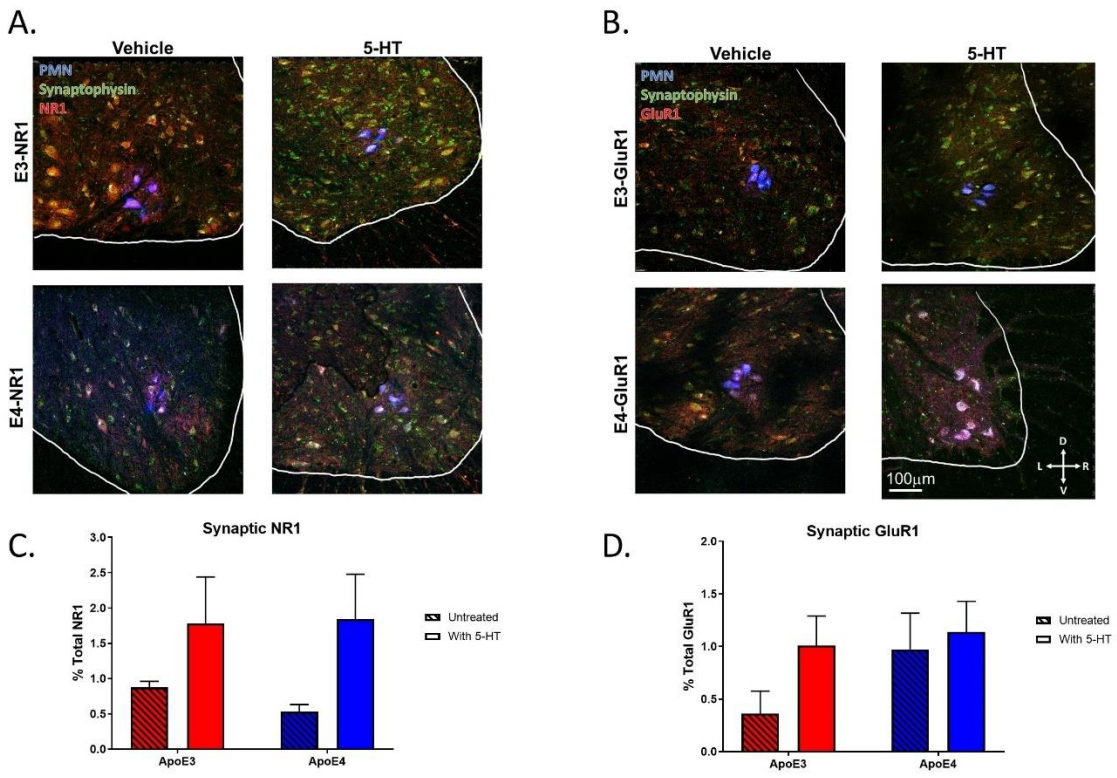

Figure 2.2 ApoE4 prevents 5-HT-induced upregulation of synaptic AMPA receptors.

A. Representative images of the C4 ventral horns with staining of synaptophysin (green) and NMDA receptor subunit NR1 (red) on labeled PMN (blue). Ventral horns are outlined in white. *B.* Representative images of synaptophysin (green) and the AMPA receptor subunit GLuR1 (red) on labeled PMN (blue). *C.* Quantification of the colocalization of synaptophysin and NR1. Neither E3 or E4 treated animals significantly increased synaptic NR1 in response to episodic 5-HT (2-way ANOVA p=0.21) and there was no difference between genotypes (2-way ANOVA p=0.868). *D.* Quantification of synaptic GLuR1 in the presence or absence of episodic 5-HT. Neither apoE isoform displayed a significant increase in synaptic GluR1 in response to episodic 5-HT (2-way ANOVA p=0.31). There was no significant difference between genotypes (2-way ANOVA p=0.731).

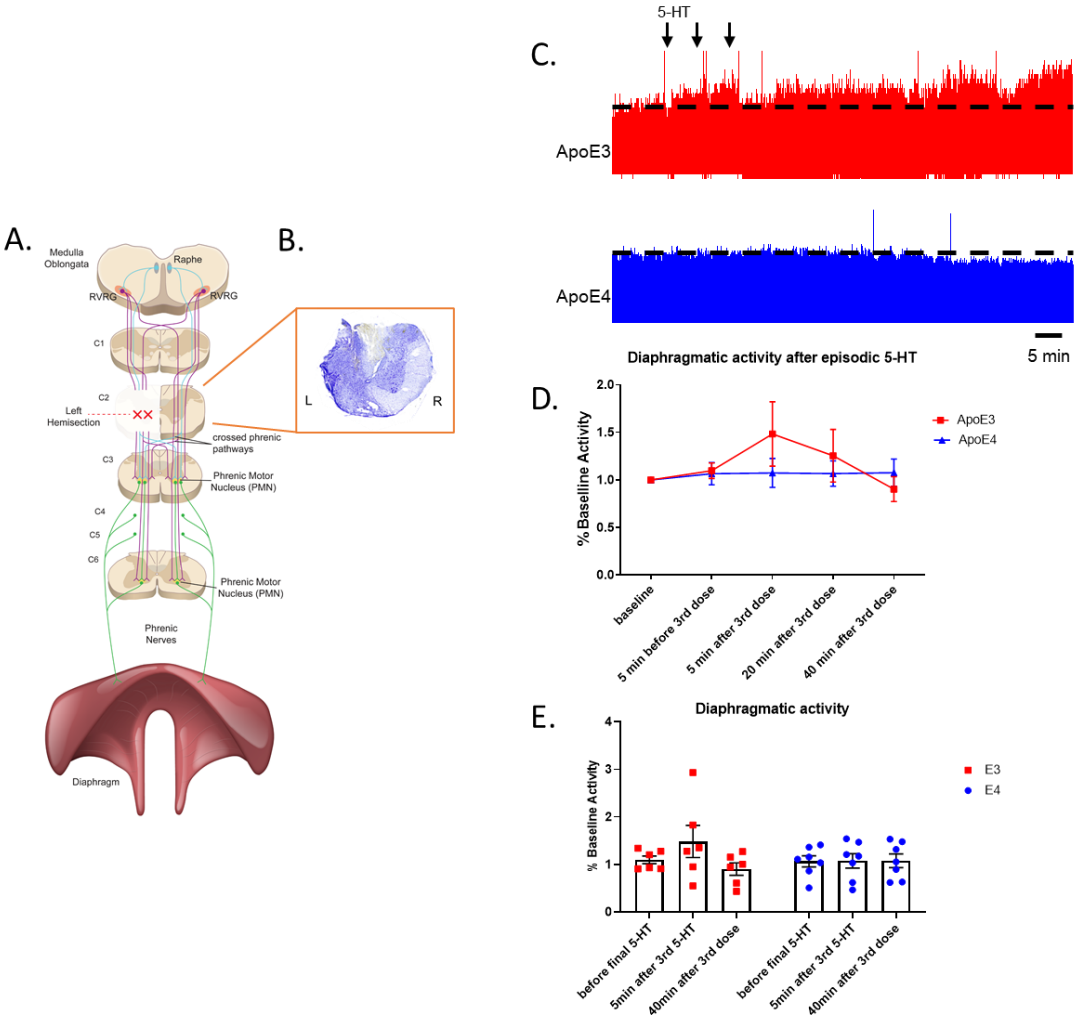

Figure 2.3 ApoE4 is inhibitory to respiratory motor plasticity after SCI.

A. Schematic of the left lateral C2 hemisection injury model and its impact on the breathing circuitry. *B.* Representative image of the rat spinal cord at the C2 level after C2 hemisection stained with cresyl violet. *C.* Representative EMG traces from injured rats. Traces show activity of the hemidiaphragm ipsilateral to injury. Arrows indicate the timing of application of the three 5-HT doses. Dashed lines indicate baseline amplitude. *D.* Quantification of diaphragmatic burst amplitude in the left hemidiaphragm of injured animals in response to episodic 5-HT. There was no significant difference in the change in activity over time between genotypes (RM ANOVA, p=0.58). *E.* Quantification of diaphragmatic EMG amplitude 5 minutes before and 5 minutes and 40 minutes after the final dose of 5-HT (Student's t-tests, E3xE4 5min before final dose p=0.83, E3xE4 5 min after final dose p=0.27, E3xE4 40 min after final dose p=0.40)

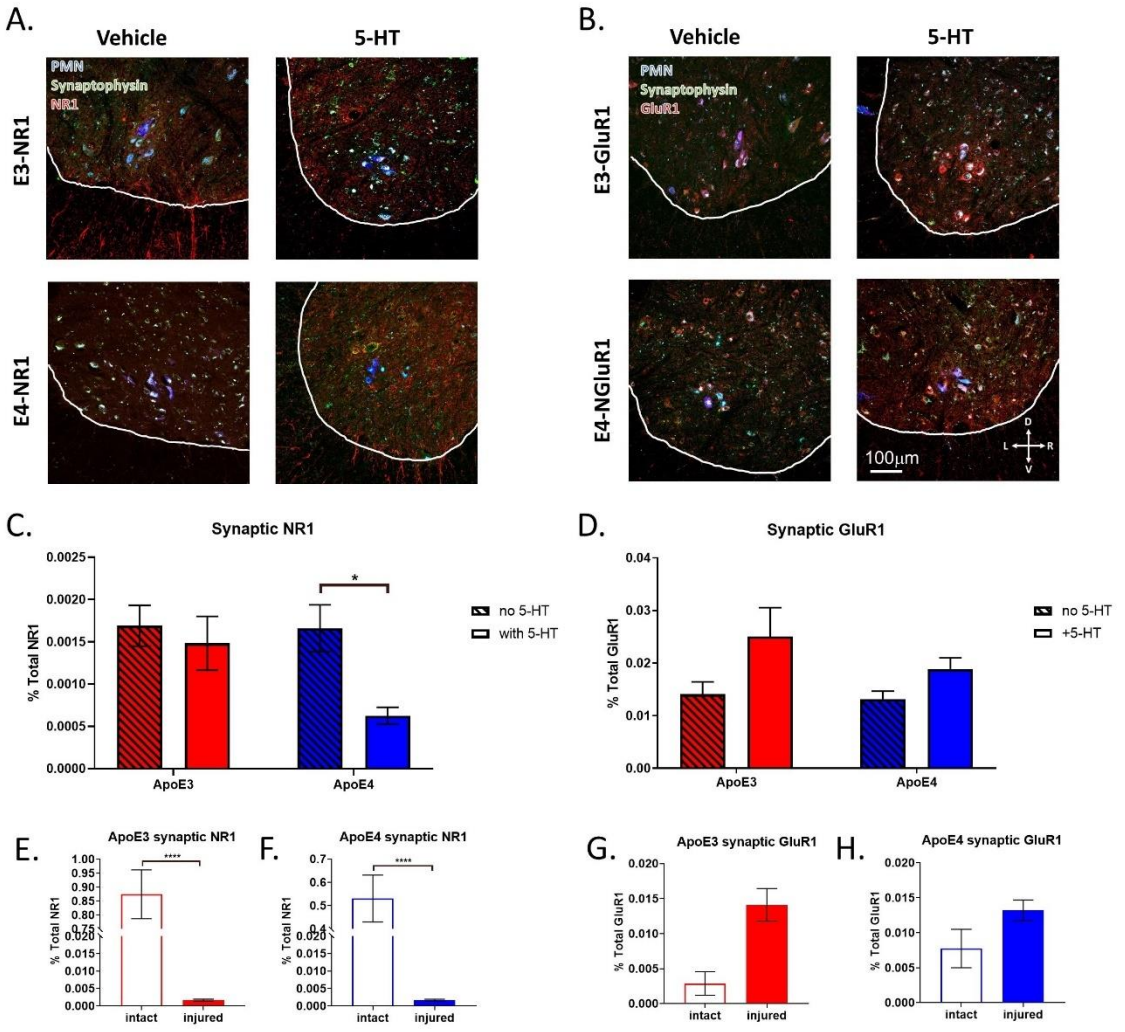

Figure 2.4 Human apoE4 decreases synaptic glutamate receptor expression after episodic 5-HT in hemisected animals.

A. Representative images of staining for colocalization of NR1 (red) and synaptophysin (green) on phrenic motor neurons labeled with CTB (blue). Ventral horns are outlined in white. *B.* Representative images of spinal cord sections with CTB-labeled PMN (blue) stained for GluR1 (red), synaptophysin (green). *C.* Quantification of synaptic NR1 colocalized with synaptophysin normalized to total intracellular NR1. There was no significant difference between ApoE3 and E4 animals (a priori 2-way ANOVA p=0.0727). ApoE4-treated animals had significantly less synaptic NR1 in response to episodic 5-HT than those that did not receive 5-HT (a priori 2-way ANOVA and Tukey's post hoc test p=0.0.0196). *D.* Quantification of colocalization of GluR1 and synaptophysin normalized to total cellular GluR1 in PMN. There was no significant difference between E3 and E4 (2-way ANOVA p=0.269). E3-treated animals exhibited a trend of increased synaptic GluR1 expression in response to 5-HT (2-way ANOVA and Tukey's post hoc test p=0.075). *E-H.* Comparisons of synaptic glutamate receptor subunit levels in intact and C2 hemisected mice that did not receive 5-HT. In both E3 and E4 animals, NR1 decreased significantly in response to injury (*E.*, Student's t-test p<0.0001

and *F.* $p<0.0001$, respectively). Levels of synaptic GluR1 were not significantly different between intact and injured animals in the presence of either apoE isoform (*G.*, apoE3 Student's t-test $p=0.29$ and *H.*, apoE4 $p=0.41$).

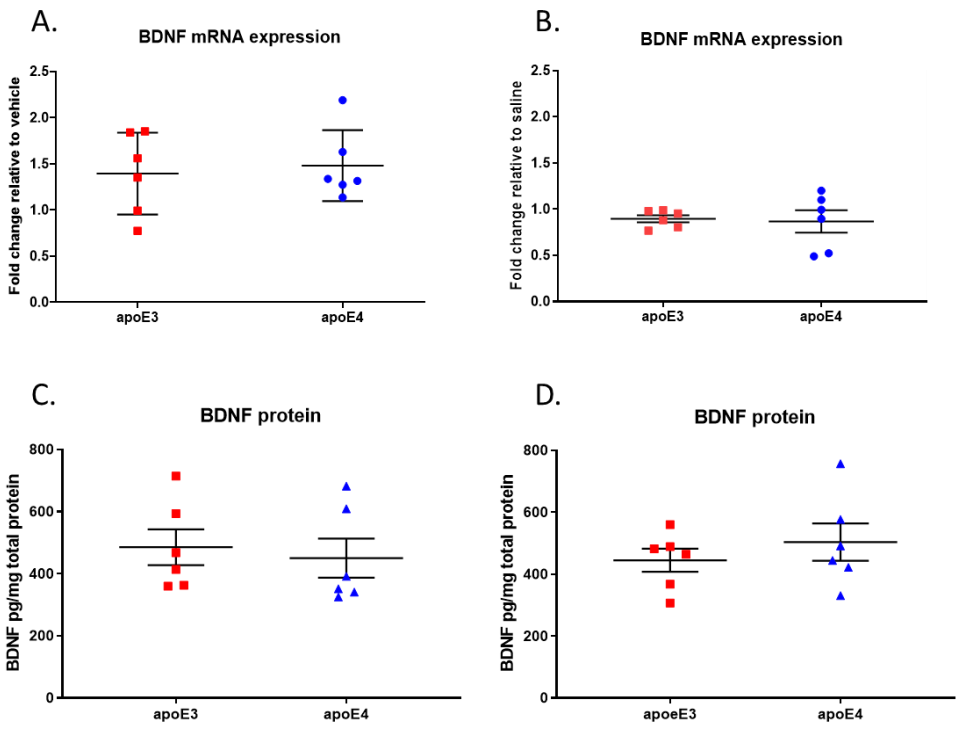

Figure 2.5 BDNF expression isn't apoE isoform-dependent.

A,B. Expression of BDNF mRNA in the region of the phrenic motor nucleus in uninjured (*A*) and injured (*B*) rats. There was no significant difference between genotypes in the presence or absence of injury (Student's t-test, uninjured p=0.73, injured p=0.83). *C,D.* Quantification of BDNF protein. There was no significant difference between genotypes in uninjured (*C*, Student's t-test, p=0.69) or injured (*D*, Student's t-test, p=0.43) animals.

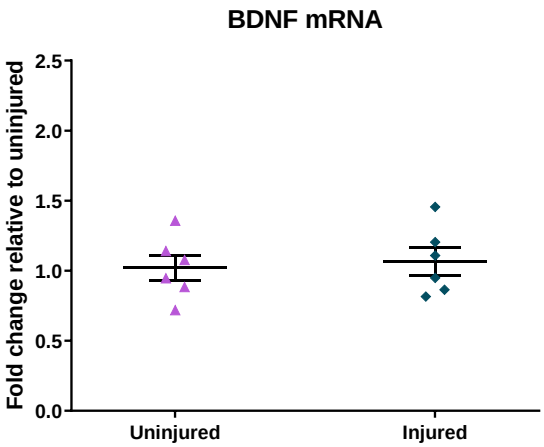

Figure 2.6 Cervical SCI alone does not change BDNF levels in the phrenic motor nucleus.

BDNF mRNA expression did not significantly differ between intact and C2 hemisected rats that were not exposed to human apoE protein (Student's t-test p=0.74)

CHAPTER 3. NOVEL INFLUENCES OF SEX AND *APOE* GENOTYPE ON SPINAL PLASTICITY AND RECOVERY OF FUNCTION

3.1 Introduction

Over 17,000 Americans experience a spinal cord injury every year.[2] Depending on the level of injury, damage to neural pathways in the spinal cord can lead to a multitude of sensory deficits and loss of crucial motor functions. Over the past few decades, many promising therapeutic approaches have been developed to enhance neuroprotection or induce anatomical and functional plasticity of spinal pathways to restore function.[139,418–421] Moreover, pivotal studies using nerve grafts, PTEN deletion, NOGO inhibition, or degradation of the perineuronal net or chondroitin sufate proteoglycans (CSPGs) have demonstrated that the CNS is capable of overcoming neural intrinsic and extrinsic barriers to regeneration after injury, leading to meaningful preclinical recovery.[93,114,116,422,423] However, these therapeutic strategies have met with varied clinical success and there remains a lack of effective treatment strategies for the human SCI population.[424]

A striking difference between individuals living with SCI and the animals used to model them is the level of genetic diversity represented in these populations. In contrast to the incredible diversity of the human population, preclinical studies typically utilize homogenous groups of animals with the same sex and similar genetic backgrounds. While this does facilitate easier determination of treatment effects, it also makes it less likely that discoveries in these models will translate to human patients. Although an increasing number of preclinical investigations are addressing how sex influences the efficacy of therapeutic strategies, the impact of genetic variability remains largely

unexplored. A recent review by Fouad et al. specifically outlined the importance of evaluating the influence of factors such as sex and genotype to address the neuroanatomical-functional paradox and lack of therapeutic translation in SCI.[377] Indeed, we hypothesize that genetic factors could play a considerable role in determining how individuals respond to treatment strategies.

Apolipoprotein E (ApoE) is a highly expressed lipid carrier in the CNS.[303] It is encoded by the *APOE* gene, which exists in three common alleles designated ε2, ε3, and ε4. The ε4 allele, which is carried by nearly 1 in 5 individuals, has been associated with a number of detrimental outcomes, including a weakening of synaptic plasticity in the brain.[425] However, despite a robust body of literature in neurodegenerative diseases and traumatic brain injury[347,350,426], the impact of ε4 on plasticity in the spinal cord remains underexplored. We hypothesized that spinally-mediated plasticity is constrained in apoE4 animals, thereby demonstrating the importance of considering the diversity of the human population when developing therapeutic approaches for people with SCI.

To test this hypothesis, we utilize a model of cervical injury to examine how *APOE* genotype alters the response to intermittent hypoxia (IH). Most SCIs occur at these high levels and can disrupt the neural circuitry that mediates breathing, leading to respiratory insufficiency and potentiating the need for mechanical ventilation.[2,427,428] Mechanical ventilation increases the risk of respiratory infection, a leading cause of rehospitalization and death following cervical spinal cord injury (cSCI).[402]

In recent years, there has been a growing appreciation for the potential of intermittent hypoxia (IH) as a treatment strategy for a host of conditions including SCI.[230]

In clinical trials, therapeutic IH has been utilized to increase limb function and to facilitate ventilation in persons with SCI by enhancing plasticity in the spinal cord.[140,142,381] Neural pathways in the cervical region which mediate breathing are critical therapeutic targets of IH, including spared pathways which might remain after injury. However, the influence of human genetic variability on IH-induced recovery is unknown. Therefore, we utilized this model of spinally-mediated plasticity to examine how expression of different human *APOE* alleles alter the efficacy of therapeutic strategies, such as IH, that are being developed to enhance plasticity following SCI. Our results provide evidence that both sex and *APOE* genotype determine the propensity for plasticity in humanized mice that are exposed to therapeutic IH.

3.2 Methods

This chapter of the Dissertation has been adapted from a manuscript published in *eNeuro*.[429]

3.2.1 C2 Hemisections

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 mouse *APOE* promotor (targeted replacement mice) were backcrossed for at least 10 generations to the C57BL/6 background.[398–400] Mice were group-housed on a twelve-hour light/dark cycle and fed normal chow diet *ad libitum.* All mice were 92-105 days old at the time of injury. Female (20-24g) and male (22-30g) mice were anesthetized with isoflurane. Animals were then prepped for surgery by shaving the surgical area followed by disinfecting with alternating betadine and 70% ethanol swabs. Puralube

ophthalmic ointment was applied to the eyes to prevent drying during surgery. A midline incision was made through the skin just caudal to the ears to between the scapulae. Marcaine/bupivacaine was instilled along the incision site. The acromiotrapezius, semispinalis capitus, and rectus capitus posterior muscles were cut along their midline, bluntly dissected, and retracted. Paravertebral muscles were cut away from the C2 vertebra using ToughCut Spring Scissors (Fine Science Tools). The lamina of the C2 vertebra was then removed using Spring Scissors. Under a dissecting microscope (Meiji EMZ), a left lateral C2 hemisection (C2Hx) was performed by inserting a 27-gauge needle into the midline of the spinal cord at the C2 level and dragging the needle to the left lateral edge of the cord. This was then repeated once to ensure a complete injury. Musculature was sutured (6-0 absorbable suture) and skin was closed with Vetbond Tissue Adhesive (3M). Animals received subcutaneous buprenorphine (0.75mg/kg) and carprofen (10mg/kg) immediately after surgery. Male mice were housed individually following surgery to prevent fighting amongst cagemates.

3.2.2 Intermittent Hypoxia and Diaphragmatic Electromyography (EMG)

Three weeks after hemisection, animals were anesthetized with isoflurane using the SomnoSuite Anesthesia System (Kent Scientific). A laparotomy was performed by cutting through the rectus abdominis, external oblique, and internal oblique muscles. Bipolar electrodes, connected to an amplifier and data acquisition system (CWE BMA-400 Four-channel Bioamplifier, CED 1401 with Spike2 Data Analysis Computer Interface), were inserted into the dorsal region of the left hemidiaphragm, where they were secured using Vetbond. Bilateral recordings were not performed due to the

increased attrition rate we observed after performing bilateral electrode insertion. The laparotomy was also closed using Vetbond. Ten minutes of baseline breathing activity was recorded. The air input to the Somnosuite was then changed from room air (normoxia) to a tank of 11% oxygen, 89% nitrogen gas (hypoxia) for 5 min, at which point it was switched back to room air for 5 minutes. This was repeated for 3 bouts of hypoxia separated by 5 minutes of normoxia. Diaphragmatic activity was recorded for 1 hour after the final hypoxic bout (Fig. 1 *A*). Although core temperature was not monitored during recordings, animals were kept on heating pads throughout all recording procedures to maintain body temperature.

3.2.3 Sectioning and Staining

To harvest tissue for immunohistochemistry, mice were perfused with 4% paraformaldehyde (PFA) following the diaphragmatic EMG recording. The spinal column was isolated and placed in 4% PFA at 4°C. After two days, tissue was removed from PFA and placed in a 30% sucrose solution at 4°C for cryoprotection until sectioning.

Tissue was mounted and frozen in Tissue Plus O.C.T. Compound (Fisher Healthcare) and cut at a thickness of 20μm on a cryostat (Leica). Serial sections from the injury site (C1-C2) were placed on one set of slides while serial sections from the level of the PMN (C3-C6) were placed on another set. Injured tissue slides were dehydrated in ethanol and stained with 0.1% Cresyl violet solution (Sigma Cat #C5042). Slides were then mounted using permount (Electron Microscopy Sciences Cat #17986-01). For 5-HT staining, frozen section were thawed to room temperature, rinsed with 1x PBS, and

blocked in a solution of 5% normal goat serum, 0.1% bovine serum albumin, and 0.1% TritonX-100 dissolved in PBS. Slides were incubated in 5-HT primary antibody diluted 1:10,000 (rabbit, ImmunoStar Cat #20080) then goat anti-rabbit AlexaFluor488 secondary antibody (1:500, Life Technologies Cat #A11034). Stained slides were mounted with ProLong Gold mountant with DAPI (Invitrogen Cat #P36931). For WFA (Wisteria Floribunda Lectin) staining, frozen sections were thawed to room temperature, washed with 1xPBS, then blocked in 3% normal goat serum diluted in PBS. Slides were then incubated in WFA primary antibody conjugated to Fluorescein at a dilution of 1:400 (Vector Labs Cat #FL-1351). Stained slides were mounted in ProLong Gold with DAPI.

3.2.4 Trace Analysis

After recording, raw diaphragmatic EMG was rectified and integrated using Spike2 software. Analysis was performed at twelve time points: twice during baseline recording, once during each hypoxic and normoxic bout, and at 10, 20, 30, and 40 minutes after the final hypoxic period (Figure 3.1 *A*). For each time point, peak amplitude was averaged over a 30 second period. Amplitude of diaphragmatic bursts at each time point were normalized to that animal's pre-hypoxia baseline amplitude. Frequency of diaphragmatic bursts, indicative of breaths, was also quantified over a 30 second period at each time point.

3.2.5 Imaging and image quantification

Staining for cresyl violet and WFA was imaged on a Keyence BZ-X810 fluorescence microscope for quantification. Cresyl violet stained sections were imaged

using brightfield illumination at 2x. Sections stained for WFA were imaged at 10x using the monochromatic camera with high resolution (0.75488μm/pixel) for quantification. Additional images for publication were acquired on a Nikon Eclipse T*i* series inverted confocal at 40x, focused on the ventral horn in the region of the putative PMN. Sections stained for 5-HT were imaged on the Nikon at 20x. Images of 5-HT staining for publication were taken at 40x in the region of the PMN. All imaging and quantification was performed on the ventral horn of the left side of the cord, ipsilateral to the injury.

WFA labeling was quantified using the HALO image analysis platform (Indica Labs). We developed and optimized an algorithm on the Area Quantification v1.0 to capture positive staining for WFA while omitting any nonspecific fluorescence. A region of interest (ROI) was drawn around the left ventral horn of sections at the level of C4. The quantification algorithm was applied to the ROI of each section. The area of staining was then normalized to the total area of the ROI. Three tissue sections at level C4 were analyzed for each animal. 5-HT labeling was also quantified with HALO. A region of interest was drawn around the left ventral horn. Serotonergic fibers within the ROI were traced using the embedded annotation tool. The total length of fibers was then normalized to the area of the ROI.

3.2.6 Experimental Design and Statistical Analyses

Sample sizes for mice receiving diaphragmatic EMG recordings were calculated based on preliminary data from 10 hemisected mice representing all three genotypes using Cohen's D to estimate effect size (data in Table 3.2). Group sizes for each sex and genotype are found in Table 1. Tissue from a subset of animals was perfused with PFA

and spinal cord tissue was harvested from these animals for IHC and quantification of WFA and serotonergic sprouting (apoE3 n=4, apoE4 n=5)

For statistical analysis of EMG traces, repeated measures (RM)-ANOVA was used to account for within-subject correlation given repeated measurements over time. Stratified RMANOVA analyses were performed on male and female traces. Results were considered statistically significant if $t \geq 1.96$. Student's t-test was used to analyze 5-HT fiber staining on spinal cord tissue. Welch's t-test for unequal variances was used to analyze staining of WFA. Results were considered statistically significant if $p < 0.05$. Investigators were blinded until all diaphragmatic EMG and histology analyses were complete. The mean difference (MD) and 95% confidence interval (CI) were calculated to provide an estimate of the range of possible differences between groups.

Table 3.1 Injured group sizes

	Male	Female
E3	n=7	n=8
E4	n=6	n=11

3.3 Results

3.3.1 Respiratory motor plasticity in C2 hemisected humanized *APOE* mice

At 3 months of age, male and female mice received a left C2 hemisection by making an incision from the midline to the left lateral edge of the spinal cord just caudal to the C2 dorsal roots. This injury effectively disrupts the neural circuitry that descends from the ipsilateral medullary respiratory nuclei to phrenic motor neurons on the left side (Fig. 3.1 *B).* At the time of injury, hemisection was visually confirmed by observing the

thorax of each mouse to ensure that only the right side of the thorax continued rhythmically expanding with each breath. Injury completeness was histologically confirmed upon sacrifice of a subset of mice (n=16) using cresyl violet (Fig. 3.1 *C*). All mice were homozygous for human ε3 or ε4 alleles expressed under control of the murine *APOE* promoter as described previously.[398,400] At 3 weeks post-injury, mice were exposed to intermittent hypoxia. This consisted of 3 hypoxic (11% O_2) bouts of 5 minute duration separated by 5 minutes of normoxia as illustrated in Figure 1. We evaluated the breathing response to IH by concurrently recording diaphragmatic EMG, which continued for 1 hour following the final hypoxic bout. Amplitude of diaphragmatic bursts was quantified while blinded and then grouped according to *APOE* genotype. No difference was found in the response to IH between mice expressing ε3 or ε4 (Fig. 3.1 *D*, RMANOVA p=0.741). All animals appear to experience an initial decrease in diaphragmatic activity during the first hypoxic bout. Breathing in both apoE3 and apoE4 mice remained constant once the IH protocol ended (Fig. 3.1 *D*).

Previous studies in rodents have shown that IH treatment gives rise to an augmentation of breathing activity that characterizes LTF.[224,232,387,388] We therefore compared amplitude at the beginning of IH and 40 minutes after the final bout of hypoxia to determine whether breathing activity increased in response to IH. Neither genotype exhibited significant augmentation of diaphragmatic activity at 40 minutes post-hypoxia, indicative of a lack of LTF in the humanized mice (Fig. 3.1 *E*, paired t-test E3 p=0.741, E4 p=0.405).

3.3.2 Sex differences in *APOE* modulation of LTF

To investigate sex-dependent influences of *APOE* genotype on LTF, we separated data from males and females for independent analysis. Animals were weighed every day for the first 4 days after injury and then once a week until diaphragmatic EMG recordings were performed. When comparing weights over time after injury, there was no significant difference between genotypes in male (p=0.16) or female (p=0.65) mice (data not shown). Figure 3.2 *A* shows representative traces from male apoE3 and E4 mice. As evidenced in these traces, both genotypes exhibited a decrease in frequency over time after IH. However, there was no significant genotype effect on the magnitude of this decrease (Fig. 3.2 *A*, supplementary Fig. 3.1 *A*, RMANOVA, p=0.846). Previous studies in rats have reported no spontaneous recovery in the paralyzed hemidiaphragm even chronically after C2 hemisection.[218] In contrast, the overwhelming majority of the 32 mice used in the current study showed spontaneous functional recovery of the paralyzed mouse hemidiaphragm. Considering all males and females from which we recorded diaphragmatic EMG's, only 2 mice displayed no spontaneous recovery: 1 male of each genotype (Fig 3.2 *B*). Quantification of the diaphragmatic EMG data demonstrates that males expressing the ε3 allele display a decline in the amplitude of diaphragmatic bursting beginning in the first hypoxic bout and persisting throughout the recording period (Fig. 3.2 *C*). Although this deterioration of activity did not reach statistical significance (RMANOVA, t=0.03), it is worthwhile to highlight how the apoE3 males diverged from apoE4, which demonstrate slightly heightened activity at 40 minutes post-IH (Fig. 3.2 *C,D*, RMANOVA, t=-0.65).

Analysis of diaphragmatic EMGs in female mice of both genotypes showed a similar negative trend in the breathing frequency induced by IH (Fig. 3.3 *A*, supplementary Fig. 3.2, RMANOVA, p=0.673). A subset of mice displayed a complete loss of diaphragmatic activity following hypoxic exposure. We refer to these animals as "non-responders". Three non-responders emerged in the apoE4 group, while none were observed in the mice that expressed ε3 (representative trace shown in Fig. 3.3 *B*). However, unlike the male mice, all females demonstrated spontaneous recovery prior to IH (data not shown). Quantification of diaphragmatic burst amplitude in females that maintained diaphragmatic activity after IH showed that apoE3 mice responded to IH with an initial decrease in burst amplitude. This decline was temporary and activity returned to near baseline levels by 40 minutes (Fig. 3.3 *C*). However, apoE4 females exhibited an immediate reduction in burst amplitude that is still evident the end of the recording period. At 40 minutes post-IH, breathing of apoE4 females is significantly depressed compared to that of apoE3's at the same time point (Fig. 3.3 *C,D*, mixed model RMANOVA t=2.08). Consistent with the combined data, none of the female mice expressing human *APOE* developed the gradual and prolonged breathing augmentation that is characteristic of LTF.

When animals are challenged with a brief bout of hypoxia, feedback from peripheral chemoreceptors induces an augmentation of respiratory output. This change in ventilation is known as the hypoxic ventilatory response (HVR).[430] During the hypoxic bouts of IH treatment, all apoE mice exhibited a decline in diaphragmatic activity instead of the expected amplification. To further investigate the HVR in our humanized mice, we exposed an additional, smaller cohort of mice to a 10-minute bout of hypoxia and

assessed the changes in amplitude and frequency of diaphragmatic bursting. No females of either genotype displayed an increase or decrease in amplitude, but breath frequency began to decline by the end of the hypoxic period in those expressing ε4 (supplementary Fig. 3.2 *B,C*). In male mice expressing ε3, there was a sharp decline in both amplitude and frequency of diaphragmatic firing in response to hypoxia such that breathing activity was abolished at 10 minutes. Conversely, amplitude and frequency in apoE4 males remained constant during hypoxia (supplementary Fig. 3.1 *B,C*).

3.3.3 Perineuronal net upregulation and serotonergic sprouting in the phrenic motor nucleus

Secretion of chondroitin sulfate proteoglycans (CSPGs), a component of the perineuronal net (PNN), is upregulated after SCI in wild type animals, creating a barrier to plasticity, regeneration, and sprouting.[422,431] Thus far, it is unknown if the magnitude of this upregulation is modulated by human *APOE* genotype. Therefore, we utilized WFA staining to compare the amount of PNN present in injured spinal cords at the C4 level to determine if the IH-induced reduction in diaphragmatic activity observed in E4 females was correlated with increased amounts of inhibitory PNN. Indeed, we found that apoE4 females tended to have a higher density of WFA around the phrenic motor nucleus after injury, although this trend did not reach significance (Fig 3.4 *A-C,* Welch's t-test, p=0.0697).

The PNN can limit 5-HT sprouting after injury.[422] To determine whether differences in respiratory motor plasticity observed in females were due to the amount of serotonin at the level of the phrenic motor nucleus after C2Hx, serotonergic fibers were labeled and quantified in the ventral horn ipsilateral to injury. Serotonergic sprouting

after injury has previously been correlated with the restoration of breathing function and enhancement of LTF.[247] We postulated that dampened respiratory plasticity in ε4 females may be due to a lack of serotonergic sprouting after injury. Surprisingly, quantification of 5-HT+ fibers ipsilateral to injury revealed enhanced serotonergic sprouting in apoE4 females compared to apoE3 (Fig. 3.5 *A-C* Student's t-test, p=0.0193). This contradicted our expectation that a blunted respiratory response to IH would correspond with attenuated fiber sprouting after injury. Quantification of 5-HT fibers contralateral to injury showed no significant difference between E3 and E4 females, although E4 tended to have more 5-HT staining (Fig. 3.5 *D*, Student's t-test, p=0.286). After injury, E4 females had more 5-HT fibers ipsilateral than contralateral to injury, although this did not reach statistical significance (Fig. 3.5 *E*, Student's t-test, p=0.187).

3.4 Discussion

This study represents the first investigation into human genetic influences on the efficacy of experimental therapeutic strategies for SCI. Our results demonstrate that individuals' propensity for initiating beneficial neuroplastic responses to therapeutic IH is modified by sex and apolipoprotein E genotype. By utilizing a well-described model of SCI and spinally-mediated motor plasticity, we provide evidence to support the hypothesis that human genetic factors that are not represented by preclinical animal models limit the potential for recovery after SCI. Our physiology and histology data indicate that sex and genotype influence the CNS response to injury and therapeutic intervention, which poses a significant challenge to translating one-size-fits-all treatment strategies.

3.4.1 *APOE* genotype and respiratory motor plasticity

Recovery of breathing function is a top priority for people living with cervical SCI.[378] Intermittent hypoxia has promising potential to enhance spinal plasticity for the restoration of a variety of motor behaviors, including breathing.[139,404,432] Studies by Wadwha et al. (2008) and Tester et al. (2014) in human participants have demonstrated that ventilatory LTF is expressed by both male and female subjects, even when living with a chronic SCI. However, to our knowledge, the interaction of sex and genetic factors remains unexplored in the LTF literature. Preclinical studies that have addressed the impact of sex on respiratory motor plasticity revealed that sex hormone levels have significant ramifications for the potential to induce plasticity, likely due to the interaction of sex hormones and the serotonergic system.[373,433,434] Additionally, Baker-Herman et al. (2010) found that rat strains of different genetic backgrounds vary in their responses to IH, which was associated with differences in the expression of 5-HT_{2A} receptors on PMNs.

To further address how genetic variability impacts spinal plasticity, we examined the efficacy of IH for inducing LTF in targeted replacement mice expressing the human *APOE* ε3, and ε4 alleles. Since *APOE* first gained notoriety as a genetic marker for Alzheimer's Disease (AD), an extensive body of literature has investigated the impact of the apoE isoforms in the brain. The ε4 allele increases the risk of developing AD and lowers the age of onset in a dose-dependent manner.[310,312] E4 carriers display mitochondrial dysfunction, aggravated neuroinflammatory responses to CNS damage, loss of blood brain barrier integrity, and impaired synaptic plasticity.[436] These factors are

also key determinants for the extent of tissue damage, plasticity, regeneration, and the potential for recovery after SCI.[11,437–439]

APOE further became a gene of interest in our investigation after studies in human SCI patients found that people who carried the ε4 allele experienced significantly less motor recovery than non-carriers, despite spending more time in rehabilitation.[348,390] ApoE4 is known to curb recycling of NMDA and AMPA receptors to the postsynaptic membrane and reduces levels of BDNF in the CNS.[326,416,440] Since LTF requires BDNF signaling and activation of NMDA receptors, individuals expressing the ε4 allele may have a constrained response to IH.[268,384] However, our data demonstrates that mice expressing human apoE isoforms did not differ in their diaphragmatic response to IH, indicating that there may be no effect on LTF when *APOE* genotype is the sole variable being considered,

The lack of divergence between genotypes and the absence of augmented diaphragmatic activity in response to IH could also be due to metabolic changes. Many protocols for the induction of LTF, which are primarily conducted in rats, include the measurement of pCO_2 throughout recording.[221,224] This measurement provides a gauge of how metabolism is changing as a result of hypoxic hypometabolism: instead of increasing respiratory activity, small mammals respond to hypoxic conditions by downregulating their metabolic rate to reduce oxygen consumption.[441] Because we did not measure pCO_2 during EMG recordings due to the low blood volume of mice, we were unable to control for changes in metabolic rate, which could have prevented IH-induced breathing augmentation. However, we do not think that hypometabolism was responsible for

masking genotype effects since differences emerged when animals were grouped according to sex.

Interestingly, mice displayed a decrease in ipsilateral hemidiaphragmatic activity during hypoxic bouts, instead of the heightened activity that is typical of the HVR observed in rats.[430] Very little data is available on the respiratory response to IH and manifestation of LTF in C2 hemisected mice, although Minor et al. (2006) demonstrated the presence and viability of the murine crossed phrenic pathway (CPP), the anatomical substrate that mediates LTF.[247] The few studies performed in mice are variable in IH protocols and methods of assessing LTF.[231–233] Our HVR data indicates that mice respond to bouts of hypoxia differently than rats, but further experiments are needed to characterize this phenomenon. Considering the availability of transgenic mouse models, a standardized protocol for inducing and evaluating LTF in murine models would be extremely advantageous for studying how human genes influence spinally-mediated breathing plasticity.

3.4.2 Sex effects

Another explanation for the lack of observable differences between genotypes is that they could be masked by sex effects. *APOE* has long been studied in the Alzheimer's field, where genotype influences are known to be modulated by sex. While expression of the ε4 allele increases the risk of developing AD in both males and females, this risk is greater in females.[442,443] In rodents, apoE4-related deficits in learning and memory are aggravated in females, indicating that synaptic plasticity in the brain is impaired in a sex-dependent manner.[336,444] The implication for similar trends in spinally-mediated plasticity

led to further analysis of our data, in which the influence of genotype was investigated separately in males and females.

Diaphragmatic EMG recordings from females revealed a significant difference between the response of apoE3 and apoE4 animals. Forty minutes after IH, diaphragmatic activity was significantly depressed in females expressing ε4. Consistent with findings in the brain, this demonstrates that apoE4 females have a limited propensity for plasticity in the spinal cord. This pattern was not reflected in male mice. In contrast with the current body of literature, we show that apoE3 males experience a barrier to synaptic plasticity, as they display the largest decrease in diaphragmatic activity.

To our knowledge, apoE3-associated attenuations of plasticity have never been reported in young adult mice. Since the majority of apoE literature describes its effects on the brain and the periphery, it is possible that our results are due to a unique action of apoE in the spinal cord. The mechanism behind induction of LTF in the bulbospinal breathing circuitry is similar to that of long term potentiation (LTP) in the hippocampus: both rely on synaptic strengthening brought about by activation of postsynaptic NMDA receptors and signaling through ERK.[384,445,446] Disparate results from a variety of studies in targeted replacement mice suggest apoE4 can be detrimental or beneficial to LTP depending on brain region, sex, and age.[331,335,447,448] Taking this into account, it is less surprising to see that apoE3 also has the potential to augment or impede similar mechanisms of plasticity. This effect may also be dependent on age and region of the CNS.

3.4.3 The inhibitory PNN and serotonergic presence after C2Hx in targeted replacement mice

Following SCI, there is a dramatic upregulation of inhibitory CSPGs at the site of injury and in denervated targets.[99,422,449] Indeed, after dorsal column transections, there is an upregulation of the CSPG-containing PNN around sensory nuclei [449] and in previous studies utilizing lateral C2 hemisections, PMNs became further encased by CSPGs and the PNN.[422] Despite the abundance of evidence implicating the importance of CSPGs in limiting plasticity, regeneration, and recovery (reviewed by Tran et al., 2018), the influence of human genetics (and *APOE* alleles) on PNN structure and neuronal sprouting in the injured spinal cord has never been investigated. However, a study of human brains indicated that apoE4 augments expression of a CSPG known as brevican in the brain of Alzheimer's patients, which could explain the more extensive staining of PNN observed in E4 mice.[451]

Indeed, our findings indicate that apoE4 females exhibit a greater density of the PNN in the ventral horn region containing PMNs after injury. Although PMNS were not discreetly labeled, upregulation of the PNN at the C4 level ipsilateral to injury suggests that deficits in respiratory motor plasticity could be a consequence of the PNN's numerous influences on CNS function and plasticity. Appearance of the PNNs containing CSPGs during development ends critical periods in which experience-dependent plasticity shapes neural circuitry. Degradation of CSPGs reopens this critical period and restores synaptic plasticity in the adult CNS.[452] Following lateral spinal hemisection, increasing densities of CSPG molecules impede calcium diffusion and block action potential conduction in intact axons that are spared by the injury.[453,454] These molecules

also create an inhibitory microenvironment that prevents sprouting and regeneration of fibers in the injured spinal cord, including 5-HT fibers that have the potential to enhance functional recovery after experimental SCI.[209,218,422]

Since serotonergic signaling at the level of PMNs is crucial to induction of LTF[224], we quantified 5-HT staining around the putative PMN in spinal cords from E3 and E4 females. Density of 5-HT fibers was higher in E4 females both contralateral and ipsilateral to injury, although this difference did not reach statistical significance on the contralateral side. This indicates that compared to apoE3, apoE4 females may have greater serotonergic innervation of the PMN in the absence of injury. However, additional studies are needed to determine whether females expressing ε4 have greater serotonergic innervation prior to injury, as well as after C2Hx. Indeed, if this pattern is consistent regardless of injury status, increased 5-HT fiber density could represent a compensatory mechanism that maintains motor neuron excitability in these animals while combatting the loss of synaptic integrity over time that is observed in E4 animals.[407] The observed attenuation of LTF after injury may therefore be due to apoE4-dependent decreases in 5-HT receptor expression on PMNs, or a result of alterations downstream of 5-HT receptor activation in the signaling pathways that are necessary for the induction of LTF.

Although the higher density of PNN in E4 females is not associated with a decrease in the amount of 5-HT at the level of the PMN after injury, the CSPG-containing PNN could still play a role in abrogating respiratory motor plasticity. Further investigations are needed to determine whether CSPGs block ion flow in spared axons such as the CPP after cervical hemisection similar to the inhibition observed after

thoracic injury.[453,454] Previous studies have shown that degradation of CSPGs leads to increased presence of 5-HT around PMNs, which is associated with recovery of breathing function. However, these studies did not address at the effect of CSPG upregulation or degradation on glutamatergic sprouting or regeneration.[209,218,422] Therefore, alterations in glutamatergic innervation of PMNs could also contribute to the enhancement of diaphragmatic function demonstrated in these studies. Although E4 females displayed more 5-HT fibers than E3 females, further examination of glutamatergic axon regeneration and sprouting, as well as how enzymatic degradation of CSPGs alters this innervation, could provide insight into whether PNN upregulation contributes to a lack of respiratory motor plasticity in females expressing ε4.

The primary goal of this study was to investigate the role of genetic variability in determining an individual's propensity for spinal plasticity and recovery of breathing function after SCI. Preclinical studies typically test therapeutic approaches in a homogenous group of animals, which does not represent the diversity found in the human population. As IH and other therapeutics enter clinical trials, their efficacy for treating a heterogeneous population is an important consideration. Overall, our findings that sex and *APOE* genotype modulate the response to therapeutic IH, along with the current dearth of successful treatment strategies for SCI, emphasizes the importance of advancing personalized medicine to improve outcomes for injured individuals.

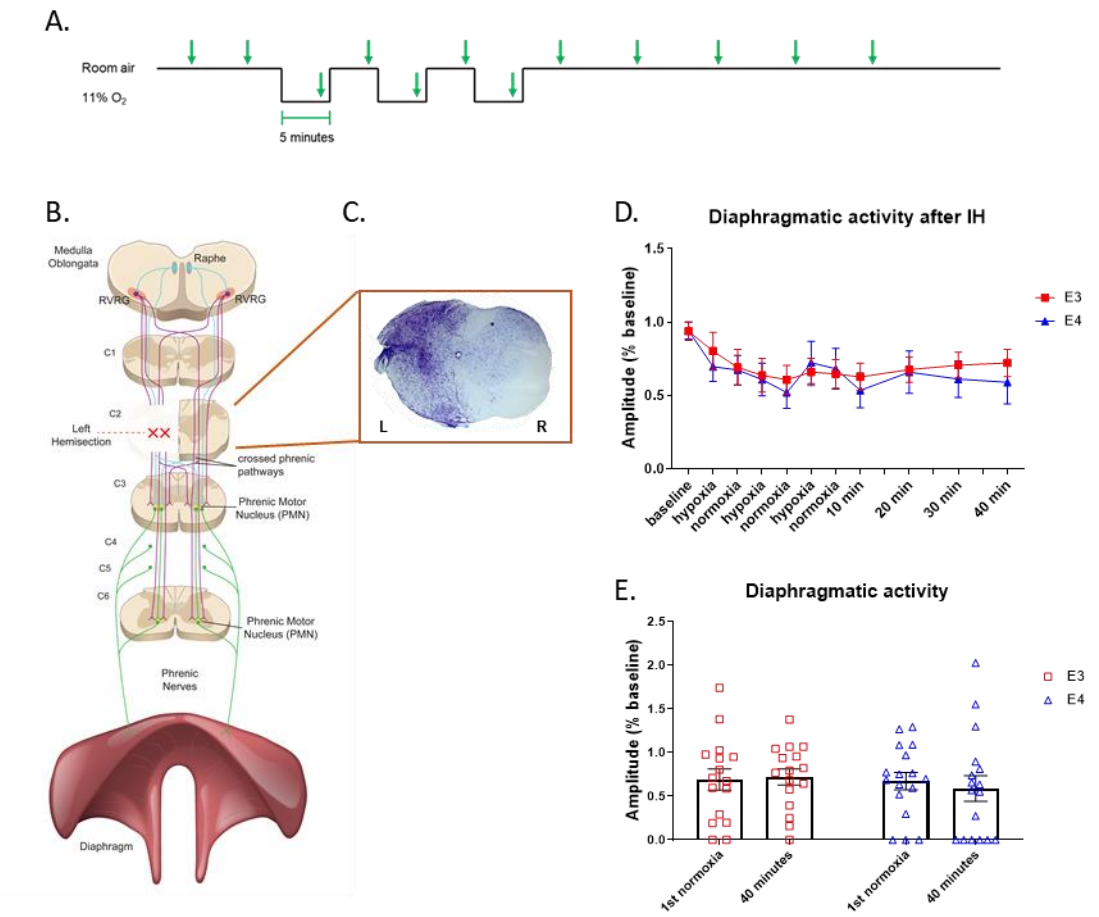

Figure 3.1 Magnitude of respiratory motor plasticity is not determined by *APOE* genotype alone.

A. Timeline of intermittent hypoxia protocol. Green arrows represent time points at which peak amplitude was analyzed. *B.* Schematic of the neural circuitry that mediates breathing. Location of the left C2 hemisection is indicated by red X. *C.* Representative image of cresyl violet staining of the spinal cord at the C2 level after injury. *D.* Quantification of diaphragmatic amplitude over time during and after IH. There was no difference between genotypes in the change in amplitude over time (RM ANOVA p=0.741) E. Quantification of diaphragmatic amplitude during the first normoxic bout and 40 minutes after IH (Paired t-test E3 normoxia v. 40min p=0.741, E4 normoxia v. 40min p=0.405)

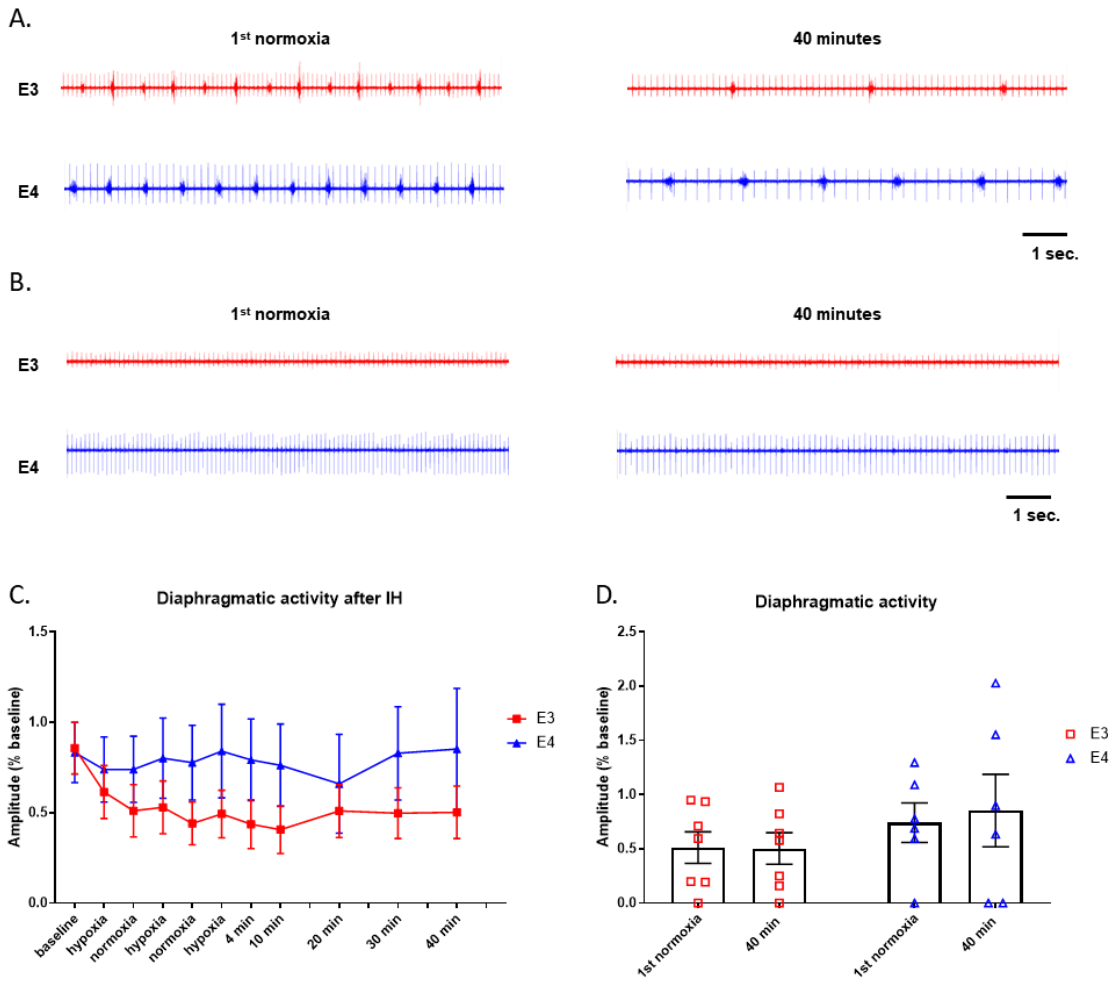

Figure 3.2 ApoE3 males demonstrate a trend of decreasing diaphragmatic activity in response to IH.

A. Representative traces of diaphragmatic activity during the first normoxic bout and 40 minutes after IH. *B.* representative traces from male mice that had no spontaneous recovery. *C.* Quantification of diaphragmatic activity over time during and after IH. Amplitude of diaphragmatic bursts is not significantly different between E3 and E4 animals. *D.* Quantification of diaphragmatic activity during the first normoxic bout and 40 minutes after IH (RM ANOVA E3/E3 t=0.03, E3/E4 norm t=-0.55, E3/E4 40 min t=-0.91, E4/E4 t=-0.67).

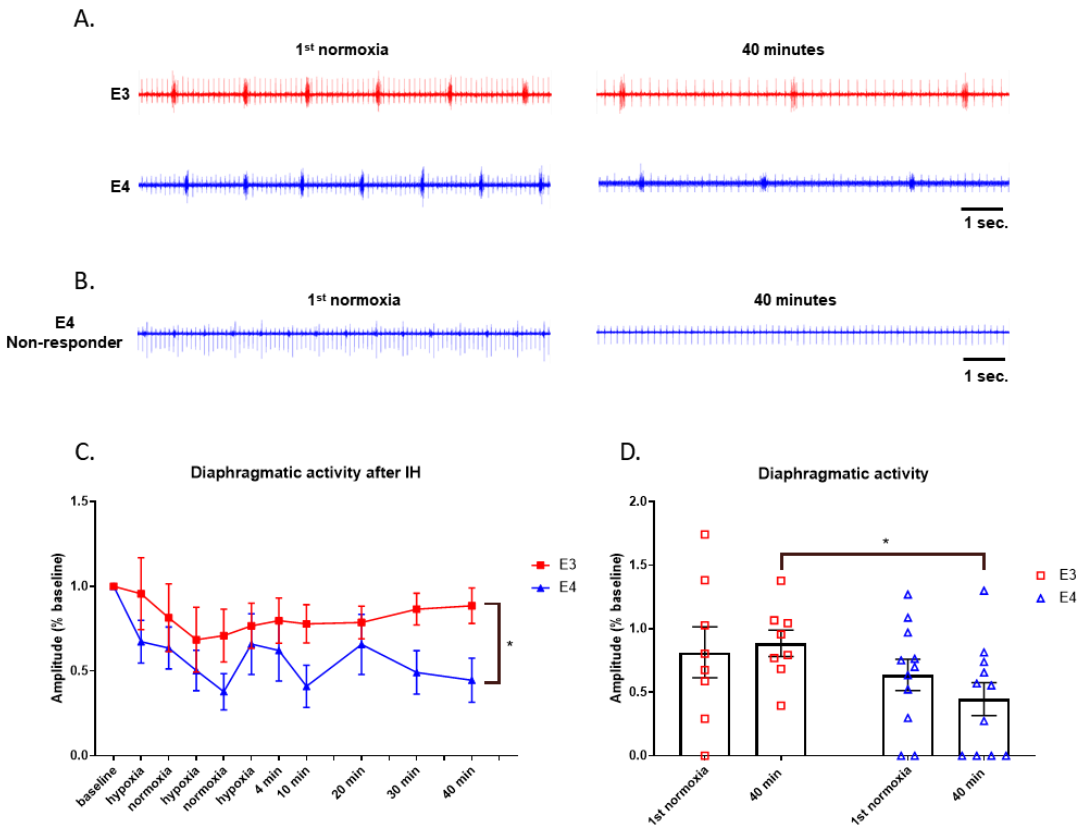

Figure 3.3 ApoE4 females display significantly less diaphragmatic activity than E3 females after IH.

A. representative traces of diaphragmatic activity during the first normoxic bout and 40 minutes after IH.*B.* representative traces from an apoE4 female non-responder. *C.* Quantification of diaphragmatic activity over time during and after IH. Amplitude of diaphragmatic bursts are significantly greater in E3 females than in E4 at 40 minutes post-IH (RMANOVA t=1.01. *D.* Quantification of diaphragmatic activity during the first normoxic bout and 40 minutes after IH (RM ANOVA E3/E3 t=-0.32, E3/E4 normoxia t=0.84, E3/E4 40 min t=2.08, E4/E4 t=1.01).

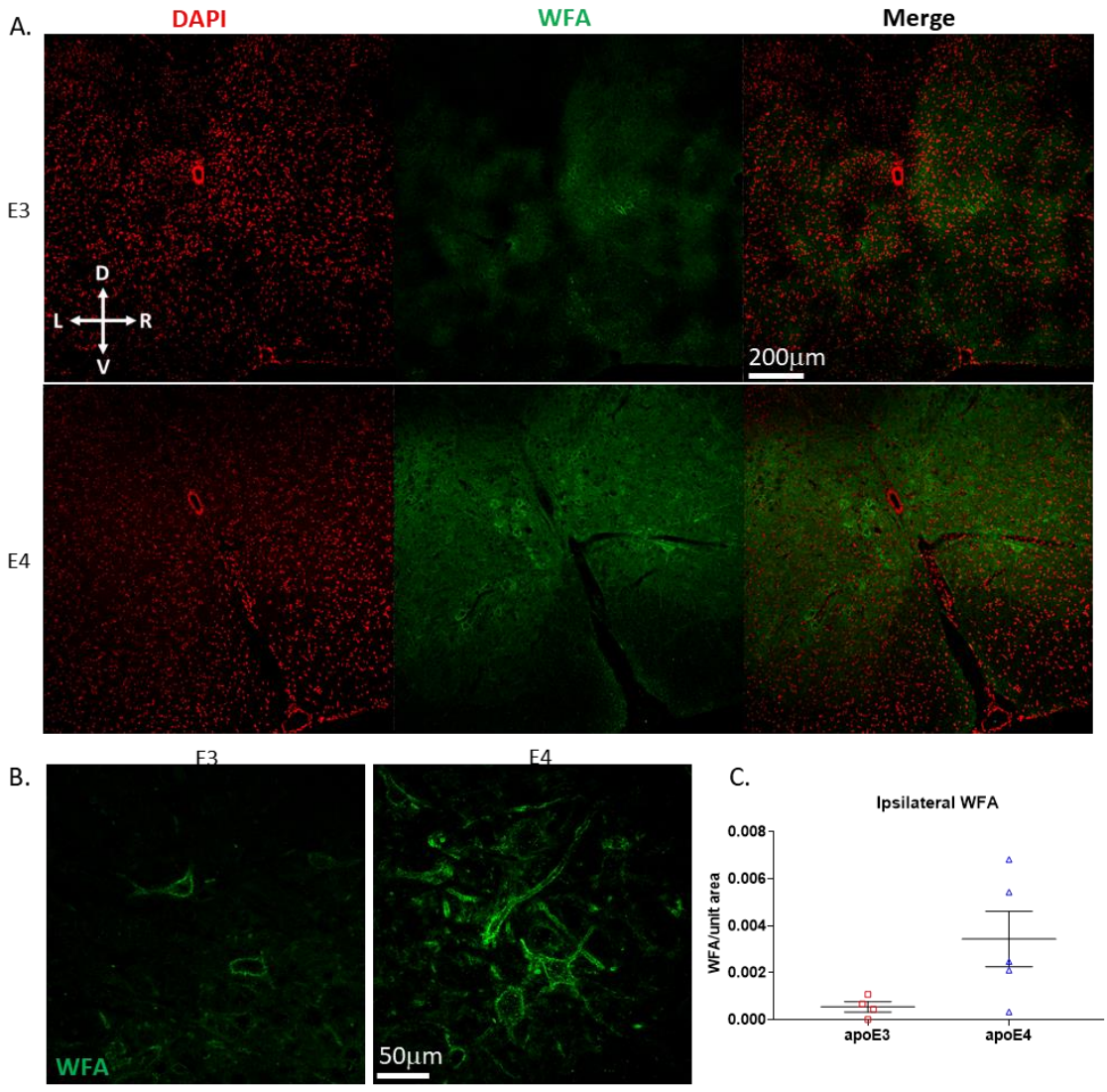

Figure 3.4 E4 females have higher levels of PNN.

A. Representative images of WFA staining at the C4 spinal cord level (DAPI is in red, WFA is in green). *B.* Higher magnification images show the PNN surrounding putative phrenic motor neurons. *C.* Quantification of WFA indicates that apoE4 mice express more WFA than E3 mice, although this trend is not statistically significant (Welch's t-test p=0.0697). Bars represent mean ± SEM.

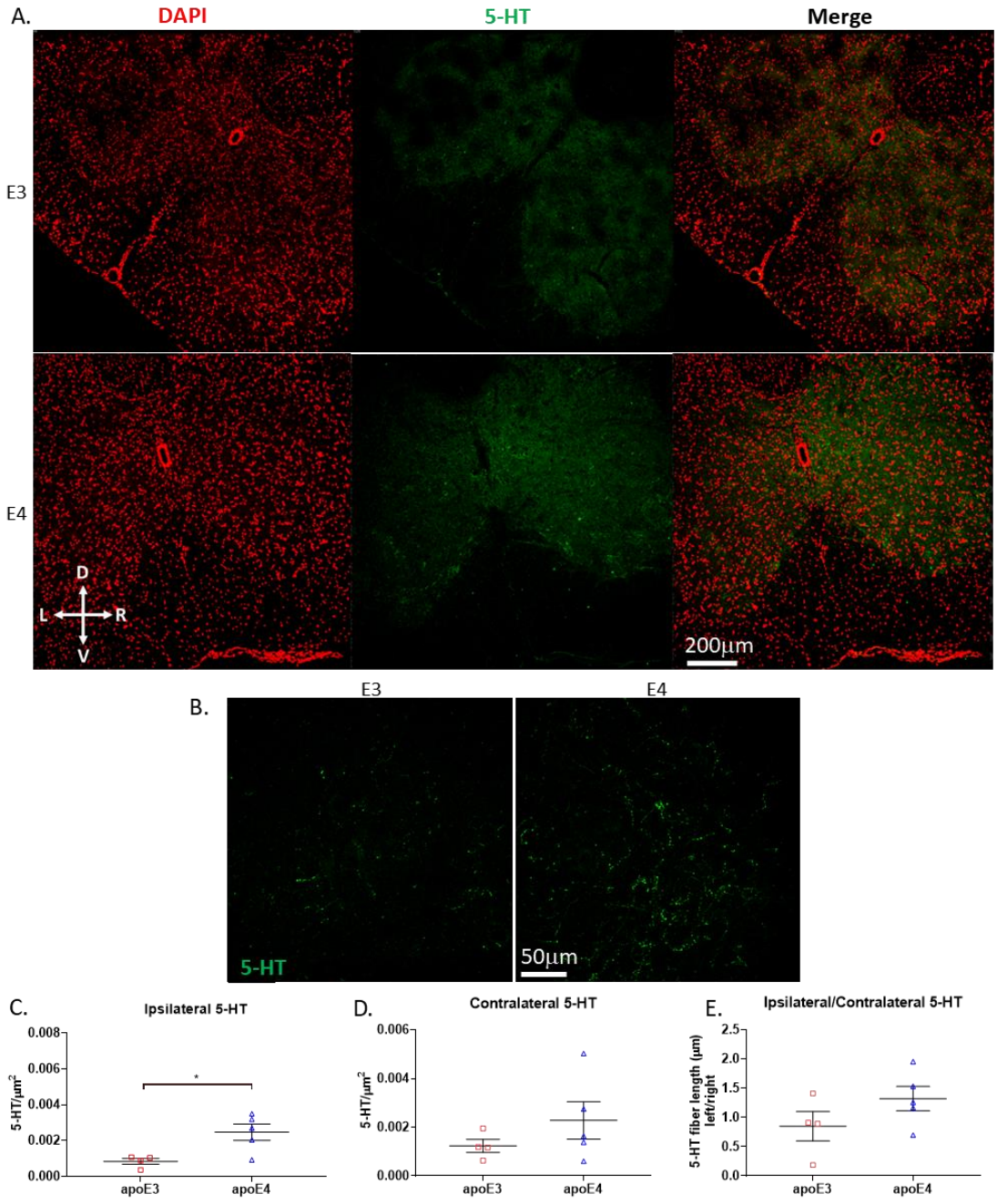

Figure 3.5 E4 females have higher density of spinal 5-HT fibers.

A. Representative images of stained 5-HT fibers in the C4 spinal cord level. Higher magnification shows individual 5-HT fibers in the area of the putative PMN. *C.* Significantly more serotonergic fibers are found ipsilateral to injury in apoE4 females (Student's t-test p=0.0193). *D.* 5-HT fibers contralateral to injury at the C4 level (Student's t-test p=0.286). *E.* Ipsilateral 5-HT staining normalized to contralateral (Student's t-test p=0.187). Bars represent mean ± SEM.

CHAPTER 4. INTERACTIONS OF SEX AND *APOE* GENOTYPE IN THE MODULATION OF RESPIRATORY MOTOR PLASTICITY

4.1 Introduction

Drawing breath is crucial for maintaining life. The respiratory system must be adaptable to changes in the environment or alterations in homeostatic signaling in order to preserve its optimal functioning at all times. The neural pathways that mediate breathing form complex and interconnected circuits throughout the brainstem and cervical spinal cord that regulate respiratory motor output in response to physiological signals from peripheral systems. This dynamic system changes throughout an organism's life as metabolic demand is altered during exercise, sleep, stress, or more gradual processes like aging.[455–457] When these experience-dependent changes in the neural control of breathing persist long after a stimulus is removed, they are considered a form of respiratory neuroplasticity.[458]

Breathing is controlled by respiratory nuclei in the brainstem.[459,460] The pre-Bötzinger complex (pre-BötC), located bilaterally in the ventral medulla, is one such nucleus that performs the crucial function of rhythmogenesis for the respiratory phases.[461] Classically, rhythmogenesis was thought to depend solely pro-Bötc neurons. More recently, however, other centers of rhythmicity such as the parafacial nucleus have been identified in the brainstem.[177,462] Neurons in the pre-BötC extend their axons across the midline to synchronize respiratory rhythm with the contralateral pre-Bötc.[159,461,463] These neurons also communicate with other medullary respiratory nuclei including the rostral ventral respiratory group (rVRG).[158] Glutamatergic premotor neurons of the rVRG project to the phrenic motor nucleus at levels C3-C6 of the spinal cord to synapse on

phrenic motor neurons (PMN).[415,464] Utilizing this circuitry, brainstem respiratory nuclei control inspiratory rhythms by determining the timing of inspiratory muscle contraction. Excitation of the PMN is additionally modulated by serotonergic projections descending from the caudal medullary raphe.[465]

Adjustments in breathing activity are imperative to maintain tissue oxygenation and prevent hypercapnia. Therefore, the neural control of breathing must be sensitive and responsive to changes in O_2 and CO_2 levels. To this end, the brainstem has local chemosensation capacity. The retrotrapezoid and parafacial nuclei sense changes in CO_2 and pH, which are communicated to the preBötC, inducing alterations in ventilation to increase pO_2.[175,466] Brainstem nuclei also respond to feedback from peripheral chemoreceptors.[170] Afferent signals from chemosensitive neurons in the carotid body are transmitted to the NTS, which communicates with the preBötC to influence breathing activity[171,467]

Decreased environmental oxygen levels lead to the hypoxic ventilatory response (HVR), consisting of an initial increase in ventilation followed by short term potentiation of phrenic nerve activity once the hypoxic bout ends.[430,468,469] In contrast, intermittent bouts of hypoxia can induce long term facilitation (LTF) through serotonin and BDNF-dependent mechanisms.[224,268] LTF is characterized by a prolonged increase in breathing motor output that endures long after cessation of hypoxia.[387] The capability of intermittent hypoxia (IH) to induce LTF has been demonstrated in human subjects and numerous animal models.[231,249,386,387] This well-characterized model of respiratory motor plasticity is a useful indicator for the capacity of the breathing circuitry to respond to the external environment. Since the ability to trigger plastic responses is an essential function

of the central nervous system (CNS), we sought to determine whether individuals' propensity for breathing plasticity may be impacted by human genetic diversity.

One gene of particular interest is *apolipoprotein E* (*APOE*), which is present in three isoforms in the human population: ε2, ε3, and ε4. Previous studies of the *APOE* alleles, which encode the apoE protein, have indicated that the E4 isoform is associated with depressions in synaptic plasticity in neuronal cell culture and in the brain.[326,331,337] However, the influence of the distinct apoE isoforms on respiratory or spinal plasticity remains unexplored. Therefore, we utilized LTF, to investigate how *APOE* genotype impacts the propensity for plasticity in the neural circuitry that controls ventilation. We hypothesize that humanized mice expressing the human ε4 allele will display a lower magnitude of LTF compared to other genotypes, indicating that genetic background predisposes individuals toward or against neural plasticity in the breathing circuitry.

4.2 Methods

This chapter of the Dissertation has been adapted from a manuscript submitted to *Respiratory Physiology and Neurobiology* for review.

4.2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Mice expressing human apoE isoforms under control of the mouse *APOE* promotor (targeted replacement mice) were backcrossed for at least 10 generations to the C57BL/6 background.[398–400] Mice were group-housed on a twelve-hour light/dark cycle and fed normal chow diet *ad libitum.* Diaphragmatic EMG recordings were performed in male and female mice at 3.5-4 months of age, at which

time C57BL/6 mice are considered mature adults.[470] Since *APOE* alleles differentially influence CNS health during aging, young adults were utilized in this study to avoid these confounds.[471–473]

4.2.2 Intermittent Hypoxia and Diaphragmatic Electromyography (EMG)

Mice were anesthetized with isoflurane using the SomnoSuite Anesthesia System (Kent Scientific). A laparotomy was performed by cutting through the rectus abdominis, external oblique, and internal oblique muscles. Bipolar electrodes, connected to an amplifier and data acquisition system (CWE BMA-400 Four-channel Bioamplifier, CED 1401 with Spike2 Data Analysis Computer Interface), were inserted into the dorsal region of the left hemidiaphragm, where they were secured using Vetbond. The laparotomy was also closed using Vetbond. 10 minutes of baseline breathing activity were recorded. The air input to the Somnosuite was then changed from room air (normoxia) to a premixed gas of 11% oxygen, 89% nitrogen gas (hypoxia) for 5 minutes, at which point it was switched back to room air for 5 minutes. This was repeated for 3 bouts of hypoxia separated by 5 minutes of normoxia. Diaphragmatic activity was recorded for 1 hour after the final hypoxic bout.

To harvest tissue for immunohistochemistry, mice were perfused with 4% paraformaldehyde (PFA). The spinal column was isolated and placed in 4% PFA at 4°C. After two days, tissue was removed from PFA and placed in a 30% sucrose solution at 4°C for cryoprotection until sectioning.

To harvest fresh tissue for molecular analysis, mice were sacrificed through overdose with isoflurane. The region of the spinal cord containing the PMN (C3-C6) was

isolated and immediately placed on dry ice. The brainstem was also preserved on dry ice for future genotyping. All tissue samples were stored at -80°C.

4.2.3 Hypoxic ventilatory response assessment

Mice were anesthetized with isoflurane and diaphragmatic EMGs were recorded as described in section 2.2. Recordings were performed for 1 hour and 40 minutes to serve as a time control for mice that underwent IH with concurrent EMG recordings. At the end of that time, mice were exposed to a 10 minute bout of hypoxia. Mice were then returned to normoxic conditions and overdosed with isoflurane.

4.2.4 Sectioning and Staining

Tissue was mounted and frozen in Tissue Plus O.C.T. Compound (Fisher Healthcare) and cut at a thickness of 20μm on a cryostat (Leica). Serial sections from the level of the PMN (C3-C6) were collected on gelatin-coated slides then placed on a slide warmer at 32°C to prevent section from washing away during IHC. For serotonin (5-HT) staining, frozen sections were thawed to room temperature, rinsed with 1x PBS, and blocked in a solution of 5% normal goat serum, 0.1% bovine serum albumin, and 0.1% TritonX-100 dissolved in PBS. Slides were incubated in 5-HT primary antibody diluted 1:10,000 (rabbit, ImmunoStar Cat #20080) then goat anti-rabbit AlexaFluor488 secondary antibody (1:500, Life Technologies Cat #A11034). Stained slides were mounted with ProLong Gold mountant with DAPI (Invitrogen Cat #P36931). For WFA (Wisteria Floribunda Lectin) staining, frozen sections were thawed to room temperature, washed with 1xPBS, then blocked in 3% normal goat serum diluted in PBS. Slides were

then incubated in WFA primary antibody conjugated to Fluorescein at a dilution of 1:400 (Vector Labs Cat #FL-1351). Stained slides were mounted in ProLong Gold with DAPI.

4.2.5 Trace Analysis

After recording, raw diaphragmatic EMG was rectified and integrated using Spike2 software. Analysis was performed at twelve time points: twice during baseline recording, once during each hypoxic and normoxic bout, and at 10, 20, 30, and 40 minutes after the final hypoxic period (Fig. 4.1 A). For each time point, peak amplitude was averaged over a 30 second period. Amplitude of diaphragmatic bursts at each time point were normalized to that animal's pre-hypoxia baseline amplitude. Frequency of diaphragmatic bursts, indicative of breaths, was also quantified over a 30 second period at each time point.

To quantify diaphragmatic activity during hypoxia as a proxy for assessing HVR, analysis was performed at 0, 5, and 10 minute time points during the hypoxic bout (Supp Fig. 1 A). Amplitude was normalized to the animal's pre-hypoxia baseline amplitude.

4.2.6 Imaging and image quantification

Staining for WFA was imaged on a Keyence BZ-X810 fluorescence microscope for quantification. Cresyl violet stained sections were imaged using brightfield illumination at 2x. Sections stained for WFA were imaged at 10x using the monochromatic camera with high resolution (0.75488μm/pixel) for quantification. Additional images for publication were acquired on a Nikon Eclipse T*i* series inverted confocal at 40x, focused on the ventral horn in the region of the putative PMN. Sections

stained for 5-HT were imaged on the Nikon at 20x. Images of 5-HT staining for publication were taken at 40x in the region of the PMN. All imaging and quantification was performed on the ventral horn of the left side of the cord, ipsilateral to the injury.

WFA labeling was quantified using the HALO image analysis platform (Indica Labs). We developed and optimized an algorithm on the Area Quantification v1.0 to capture positive staining for WFA while omitting any nonspecific fluorescence. A region of interest (ROI) was drawn around the left ventral horn of sections at the level of C4. The quantification algorithm was applied to the ROI of each section. The area of staining was then normalized to the total area of the ROI. Three tissue sections at level C4 were analyzed for each animal. 5-HT labeling was also quantified with HALO. A region of interest was drawn around the left ventral horn. Serotonergic fibers withing the ROI were traced using the embedded annotation tool. The total length of fibers was then normalized to the area of the ROA.

4.2.7 ELISA

Spinal cord tissue was homogenized in protein lysis solution (10ml of 1x RIPA with 1 tablet of Roche protease inhibitor) buffer using a Tissuemiser Homogenizer (Fisher Scientific). The supernatant was isolated and diluted 1:24 in 1x PBS for a BCA assay (Pierce, cat #23225). Once protein concentration was determined, samples were diluted 1:4 and BDNF protein was quantified using the Meso Scale Discovery BDNF Uplex Assay (cat # K1526WK-1). The Meso Scale assay was run by the Biomarker Analysis Lab in the Center for Clinical and Translational Science at the University of Kentucky.

4.2.8 Experimental Design and Statistical Analyses

Sample sizes for mice receiving diaphragmatic EMG recordings were calculated based on preliminary data from 10 hemisected mice representing all three genotypes using Cohen's D to measure effect size (same analysis as Chapter 3.2.6). Group sizes for each sex and genotype are found in Table 1. Tissue from a subset of animals was perfused with PFA and spinal cord tissue was harvested from these animals for IHC and quantification of WFA and serotonergic sprouting (n=3 for each genotype)

For statistical analysis of EMG traces, repeated measures (RM)-ANOVA was used to account for within-subject correlation given repeated measurements over time. Stratified RMANOVA analyses were performed on male and female traces. For a priori pairwaise comparisons, results were considered statistically significant if $t \geq 1.96$. Student's t-test was used to analyze 5-HT fiber staining on spinal cord tissue. Welch's t-test for unequal variances was used to analyze staining of WFA. Results were considered statistically significant if $p < 0.05$.

To analyze BDNF ELISA results, the amount of BDNF was calculated in pg/mg for each animal. One-way ANOVAs were performed to compare genotypes in female mice (ε2 n=9, ε3 n=8, ε4 n=8) and male mice (ε2 n=7, ε3 n=7, ε4 n=6). Results were considered statistically significant if $p < 0.05$.

Table 4.1 Data for sample size calculation

	Male	Female
E2	n=8	n=10
E3	n=8	n=10
E4	n=8	n=9

4.3 Results

4.3.1 ApoE isoforms and respiratory motor plasticity

Diaphragmatic EMG recordings were performed in male and female mice at 3.5-4 months of age (ε2 n=18, ε3 n=18, ε4 n=17). Mice were exposed to three bouts of intermittent hypoxia (11% O_2) while concurrent diaphragmatic EMGs were recorded from the left hemidiaphragm. Fig. 4.1 *A* illustrates the IH protocol utilized in these experiments, along with the time points at which diaphragmatic burst amplitude and frequency were quantified. Fig. 4.1 *B* highlights some of the neural pathways in the medulla and spinal cord that mediate respiratory motor control and rhythmicity. Plasticity in this neural circuitry can be enhanced by IH, leading to augmentation of diaphragmatic activity that is typically observed as an increase in burst amplitude.[224] However, instead of the expected augmentation of diaphragmatic output, mice of all genotypes displayed a decrease in burst amplitude over time after IH (Fig. 4.1 *C*., $p<0.0001$). The magnitude of this decrease did not differ between genotypes ($p=0.955$). Since mice of all genotypes demonstrated an immediate deterioration of diaphragmatic burst amplitude in response to the first bout of hypoxia, we compared amplitudes during the first 5-minute bout of normoxia and 40 minutes after the IH protocol was complete. The objective of this

comparison was to determine whether there was an IH-induced increase in breathing output after the initial decline in amplitude. There was no difference in burst amplitude between these time points within or between genotypes, as shown in Fig. 4.1 *D* (p=0.955)

4.3.2 Sex effects on respiratory motor plasticity in *APOE* targeted replacement mice

EMG data was then divided according to sex to investigate whether there were sex-dependent influences on how apoE isoforms influence breathing. Fig. 4.2 *A*. shows representative traces from male mice expressing ε2, ε3, or ε4 (n=8 for each genotype) during the first normoxic bout and forty minutes after IH. As seen in these EMG traces, breathing frequency declines over time in all three genotypes, which is quantified in Fig. 4.2 *B* (p=0.0013). The magnitude of this decrease does not differ between genotypes (p=0.210). There was also no statistically significant difference in the amplitude of diaphragmatic activity during or after IH when comparing genotypes (Fig. 4.2 *C*). However within the male apoE4 mice, there was a significant decrease in burst amplitude over time (Fig. 4.2 *D*, t=0.16) while E2 and E3 males demonstrated no change in amplitude (t=0.12 and t=0.29, respectively).

Representative traces in Fig. 4.3 *A* demonstrate how breathing in female *APOE* targeted replacement mice changes in response to IH. Similar to male mice, females also displayed a decrease in breathing frequency over time (p<0.0001, ε2 n=10, ε3 n=10, ε4 n=9), but no difference between genotypes emerged when measuring the degree of this decline (Fig. 4.3 *B*, p=0.429). Quantification of EMG amplitude revealed that females expressing ε3 experience an attenuation of breathing activity after IH treatment. By 40 minutes post IH, amplitude of diaphragmatic bursts is significantly reduced in ε3 females

compared to those expressing ε2 (Fig. 4.3 *A,C,D,* t=2.20), indicative of maladaptive plasticity in these animals.

In response to hypoxic conditions, rodents increase breathing ventilatory frequency and volume in order to compensate for decreased levels of arterial O_2 detected by peripheral chemoreceptors. This phenomenon is known as the hypoxic ventilatory response (HVR, previously described by Pamenter and Powell, 2016). In our *APOE* humanized mice, there was a consistent decrease in diaphragmatic activity in response to the first hypoxic bout during IH, instead of the expected increase. Therefore, we exposed small cohorts (n=2-3) of male and female mice to a single 10-minute bout of hypoxia (11% O_2) and quantified their diaphragmatic response to determine whether this hypoxia-induced decrease in respiratory activity is apoE isoform-dependent. Males displayed no change in frequency or amplitude of diaphragmatic output. Females likewise did not demonstrate a change in amplitude in response to hypoxia. However, E4 females did display a trend of diminishing frequency of breaths during hypoxia (Fig. 4.4 *B-E*).

4.3.3 The presence of the perineuronal net and respiratory motor plasticity

The perineuronal net (PNN), is a specialized extracellular matrix that surrounds CNS neurons that contains chondroitin sulfate proteoglycans (CSPGs). High densities of these CSPG molecules has previously been shown to interfere with neuronal signaling [453,454]. Therefore, we quantified the density of the PNN around the putative phrenic motor neurons in the ventral horn of the C4 spinal cord (Fig. 4.5 *A,B*) in female mice to determine whether the differences between genotypes observed in diaphragmatic EMG traces was correlated with the amount of PNN enveloping PMNs. Quantification of WFA

staining in *APOE* targeted replacement mice indicated that there was no difference between genotypes in the amount of PNN detected (Fig. 4.5 *C,* 1-way ANOVA p=0.5853, n=3 for each genotype).

4.3.4 Serotonin and BDNF levels in mice expressing human *APOE*

LTF is a serotonin (5-HT)-dependent form of plasticity.[224] To assess whether the divergence of respiratory responses to IH observed in females was due to apoE-dependent alterations in the amount of 5-HT present around PMNs, we quantified staining of serotonergic fibers in the C4 ventral horns (Fig. 4.6 *A-B*). There was no significant difference between genotypes when comparing the volume of 5-HT+ fibers in the vicinity of putative PMN (Fig. 4.6 *C*, p=0.182, n=3 for each genotype). However, a trend emerged of E3 females exhibiting fewer 5-HT fibers than females expressing the ε2 or ε4 alleles. Due to low n's in this experiment, future work will further investigate whether this trend persists when group sizes are increased.

Following IH, increased activation of 5-HT receptors on phrenic motor neurons leads to downstream production of new BDNF, a molecule which is both necessary and sufficient to induce LTF.[268] Although 5-HT levels around putative PMNs were not significantly different between genotypes, downstream signaling pathways could still be disrupted in E4 males and E3 females, leading to the observed maladaptive responses to IH. Therefore, we quantified downstream production of BDNF. However, measurement of BDNF protein by ELISA in the region of the phrenic motor nucleus also revealed no difference between genotypes in males (Fig. 4.7 *A*, p=0.216, ε2 n=9, ε3 n=8, ε4 n=8) or females (Fig. 4.7 *B*, p=0.137, ε2 n=7, ε3 n=6, ε4 n=6).

4.4 Discussion

The body's ability to alter breathing in response to stimuli is imperative to maintain proper tissue oxygenation in different environments or metabolic states. This plasticity in the neural circuitry that mediates breathing is therefore critical in health and disease. The current study represents the first investigation into how the three alleles of the *APOE* gene could influence individuals' capacity for this plasticity in intact animals. Understanding how sex and genetic differences alter respiratory control in an intact system will facilitate the development of approaches to enhance the ventilatory response to respiratory challenges and disease in the diverse human population. The ε2, ε3, and ε4 alleles have previously been shown to differentially alter synaptic integrity and plasticity in the brain, although the impact on spinally-mediated plasticity has hitherto remained a mystery.[407,474,475] Indeed, our results support the hypothesis that *APOE* genotype predisposes individuals towards or against respiratory motor plasticity, an effect which is further dependent upon sex.

4.4.1 Respiratory motor plasticity in *APOE* targeted replacement mice

A vast body of literature has investigated and described the respiratory response to intermittent hypoxia in rodents. What has emerged from this work is a well-described model of respiratory motor plasticity known as LTF, which is characterized by a prolonged increase in breathing output.[219,227] Although the induction of ventilatory LTF through exposure to IH is also viable in human populations, the influence of human genetic factors on the development of this plasticity remains underexplored.[142,249] By exposing *APOE* targeted replacement mice to IH and recording changes in diaphragmatic

activity, we were able to demonstrate that there were no significant differences between genotypes in the respiratory response to IH when sex was not considered as a biological variable.

The majority of previous studies examining IH-induced changes in breathing have been performed in rats, leading to optimized IH protocols that have determined how severity and duration of hypoxia changes the breathing response.[226] In contrast, few studies have addressed the mouse response to IH. The limited existing literature describes IH protocols and outcome measures that vary greatly between labs.[231–234] Due to the disparities in both approach and evaluation, it is unclear how the manifestation of LTF differs in mice and rats, as well as how that manifestation might further diverge as a result of which IH protocol is used.

An important consideration when evaluating LTF expression and the efficacy of IH is that LTF can be induced through two conflicting signaling mechanisms, known as the Q and S pathways. While the Q pathway is activated through 5-HT_{2A} or 5-HT_{2B} receptors in response to moderate IH, the S pathway is activated through adenosine 2A or 5-HT_7 receptors after severe IH.[226,476–478] The IH protocol that our mice experienced corresponded with moderate IH protocols used in rats. However, while the differences in these pathways and the crosstalk between them has been well-documented in rats, these distinctions may not be comparable in mice. Therefore, further studies are needed to determine whether our protocol is activating the Q or S pathway. If it is activating both pathways, evidence from Hoffman and Mitchell (2013), Devinney et al. (2016), and Perim et al. (2018) has verified that LTF may be prevented through cross-talk inhibition.

This could explain why mice of all genotypes did not demonstrate the expected increases in breathing after IH treatment that would be indicative of LTF.

4.4.2 Sex differences in the influence of *APOE* on respiratory motor plasticity

The importance of considering sex as a biological variable in biomedical research has been emphasized in recent years. Regarding breathing, sex determines lung volume, airway size, and the respiratory response to exercise.[481,482] Indeed, the sex hormone progesterone acts as a breathing stimulant and has been used to treat breathing disorders such as obstructive sleep apnea and chronic respiratory insufficiency.[374,483,484] Although sex hormones do not appear to alter pre-Bötc function, as demonstrated by unaltered respiratory frequency in ganodectomized animals[371], other components of the neural control of breathing are influenced by estrogen and progesterone. The serotonergic raphe nuclei, which communicate with respiratory nuclei both in the brainstem and spinal cord, express estrogen and progestin receptors. Estrogen signaling in the raphe enhances 5-HT neurotransmission, suggesting that high estrogen levels in females could augment IH-induced plasticity.[485]

Considering the impact of sex on the neural control of breathing and the importance of 5-HT signaling in inducing LTF, we divided our *APOE* targeted replacement mice into males and females for individual analysis. As in the combined analysis, EMG recordings in males demonstrated no statistically significant genotype-dependent divergence in the amplitude of diaphragmatic bursting at any analyzed timepoint. However, males expressing the ε4 allele did reveal a significant decline in

burst amplitude over time after IH compared to baseline, indicating that these males may have a maladaptive response to hypoxia-induced increases in respiratory drive.

By exposing young adult male rats to IH, Zabka et al. (2006) previously provided evidence that male rodents increase breathing motor output in response to IH. The contradictory attenuation of breathing that we observed in E4 males could be due to hypoxia-induced changes in metabolism that are exacerbated by sex and *APOE* genotype. In small rodents, hypoxia induces a decrease in metabolism in order to decrease oxygen consumption.[441] This hypoxic hypometabolism prevents the need for increased ventilation in hypoxic conditions, which could explain the lack breathing augmentation in our animals. To control for the potential of metabolism to impact the manifestation of LTF, artificial ventilation with pCO_2 and temperature sampling would be necessary to ensure that metabolism is being held constant for the duration of IH exposure. However, due to the low blood volume of mice, pCO_2 sampling throughout EMG recordings was not possible. Previous work has shown that *APOE* genotype alters glucose flux through cerebral metabolic pathways of humans and targeted replacement mice, with ε4 carriers displaying hypometabolism in various brain regions, an effect which is aggravated in males.[486–488] Less is known about how apoE isoforms differentially impact metabolism in the spinal cord and the neural circuitry that mediates breathing. Metabolic impairments in E4 males could explain why they displayed more significant decreases in breathing activity.

In contrast to male mice, female diaphragmatic burst amplitude after IH differed significantly between genotypes. Compared to ε2 females, those expressing ε3 have significantly smaller bursts by 40 minutes after IH treatment. This contradicted our

hypothesis that apoE4 would be the isoform that proved to be detrimental to spinal and respiratory plasticity. Previous studies of the *APOE* targeted replacement mouse CNS have focused on the brain due to the implication of the ε4 allele in contributing to the development of Alzheimer's Disease (AD). A vast body of literature has investigated the influences of the *APOE* alleles on neural plasticity and metabolism, especially in the areas of the brain that are most severely impacted by AD. In the hippocampus and entorhinal cortex, E4 carriers exhibit reduced dendritic spine density and synaptic plasticity, leading to learning and memory impairments.[331,489–492] Little is known about how the *APOE* alleles may influence neural plasticity in the spinal cord. However, the E3-induced deficits in respiratory motor plasticity found in females was unexpected. To our knowledge, E3 has never been shown to have inhibitory effects on plasticity. Interestingly, modulation of plasticity by apoE is age and region-dependent in the brain, as evidenced by apoE4's ability to enhance hippocampal synaptic plasticity in young mice, an effect that declines with aging.[447,448] Therefore, influences of the ε3 allele could be equally as varied and dependent upon age, region of the CNS, and sex.

The diaphragmatic response to hypoxia, representative of HVR, in female mice also exhibited genotype-dependent deviations. Although there was no significant difference in diaphragmatic burst amplitude, the breath frequency of E4 females did display a sharp decline after five minutes of hypoxic exposure, which persisted until the end of the ten-minute bout. These findings indicate that there may be genotype influences on pre-Bötc rhythmogenesis. Although this decline in breathing frequency was exhibited by females expressing ε4 after a single hypoxic bout, repeated hypoxic episodes lead to a decline in burst amplitude in females expressing ε3. This could reflect a difference in

how *APOE* genotype influences plasticity in the neural control of breathing at different neurological levels. While alterations in frequency are brainstem-mediated, differences in amplitude after IH could result from effects on brainstem respiratory nuclei or on PMNs themselves.[430]

4.4.3 *APOE*-dependent density of the PNN and the neural control of breathing

There are many possible mechanisms by which the variant isoforms of apoE may alter the neural control of breathing and impact the respiratory system's response to the environment. One potential route is through changing the density of the perineuronal net (PNN). The PNN is a specialized network of CSPG-containing extracellular matrix that appears during development to surround neurons and modulate synaptic plasticity in the CNS (reviewed by Bosiacki et al., 2019; Wang and Fawcett, 2012). Development of the PNN is dependent upon neuronal activity, with its appearance beginning as periods of synaptogenesis and myelination of the nervous system are closing.[495–497]

While the CSPG-containing PNN has important functions in maintaining synaptic homeostasis, it can also impede axonal ion transport and conduction of action potentials.[453,454] Postsynaptically, PNN molecules can prevent lateral diffusion of AMPA receptors.[498] Therefore, alterations in signaling or synaptic plasticity in the breathing circuitry could be a result of apoE-dependent variations in the density of the PNN, which leads to changes in the efficiency of neural communication. In female mice that demonstrated significant genotype-dependent differences in the respiratory response to IH, staining of the PNN around putative PMNs demonstrated no difference in PNN density between genotypes. While this could indicate that changes in PNN density are not

the cause of respiratory differences between *APOE* genotypes, further studies are needed to determine whether there could be apoE-dependent alterations in the molecular composition of the PNN. Conejero-Goldberg et al. (2015) previously established that expression of a specific CSPG known as brevican, which is implicated in brain plasticity, varies between *APOE* genotypes. This suggests that distribution of various CSPGs subtypes within the PNN could also be a source of genotype-induced modifications in the neural circuitry that mediates breathing.

4.4.4 LTF signaling pathways in *APOE* targeted replacement mice

LTF is a serotonin-dependent form of plasticity.[224] Activation of 5-HT receptors leads to downstream production of new BDNF in PMNs.[268,499] In targeted replacement mice, *APOE* genotype did not alter the levels of 5-HT or BDNF observed in the area of the phrenic motor nucleus, suggesting that differences in the breathing response to IH are not dependent on the expression of these molecules. While the disparities observed in the neuroplastic response to IH may not be due to apoE's influence on presence of BDNF or 5-HT, there could also be downstream genotype-dependent deficits in the signaling pathways that lead to the manifestation of LTF. Newly synthesized BDNF binds to the TrkB (tropomyosin receptor kinase B) receptor, which is ultimately thought to induce phosphorylation of glutamate receptor subunits in PMNs.[380,500] The manner in which isoforms of apoE may influence this downstream signaling cascade currently remains a subject of ongoing investigation. However, E4 has previously been shown to mitigate glutamate receptor phosphorylation and membrane localization in cortical neurons[326], an effect which could be mirrored in PMNs.

ApoE in the CNS is primarily synthesized and lipidated by astrocytes and isoforms of apoE differentially modulate astrocytic cell functions such as phagocytosis, glucose metabolism, and lipid homeostasis.[303,324,395,501,502] In the brainstem, astrocytes perform important oxygen sensing functions that regulate the neural control of breathing (reviewed by Gourine and Funk, 2017). Even in the absence of peripheral chemosensation, brainstem astrocytes sense local hypoxia when mitochondrial O_2 consumption decreases.[504] Inhibition of mitochondrial respiration stimulates an influx of Ca^{2+}, inducing vesicular release of ATP. In the preBötC, this astrocytic ATP release activates the hypoxic ventilatory response in order to increase breathing activity.[505] However, the impact of *APOE* genotype on this central O_2 sensing mechanism is unknown. Intriguingly, apoE4 is associated with impaired mitochondrial respiration and disruptions in Ca^{2+} homeostasis, suggesting that it could also undermine the role of central chemosensation in activating the respiratory response to hypoxia.[328,486,506] Although further studies are needed to determine how brainstem astrocytes are affected in our model, this could contribute to the lack of hypoxia-induced breathing augmentation observed in *APOE* targeted replacement mice.

4.4.5 Conclusions

Breathing function must be maintained throughout life to ensure that tissue oxygenation does not decline to deleterious levels. As people encounter hosts of diverse environments during their lifespan, their respiratory systems need to be able to quickly adapt to varying amounts of atmospheric oxygen, as well as oscillating activity levels. This requires the breathing circuitry in the brainstem and spinal cord to alter breathing

patters in response to chemoreceptor feedback. However, little is known about how genetic diversity and sex interact to determine individuals' ability to initiate this respiratory motor plasticity. Our data demonstrates that both sex and *APOE* genotype dictate the respiratory response to hypoxia, thereby establishing that a subset of the population may not have beneficial responses to respiratory challenge. This data could have important repercussions for individuals who are often faced with hypoxic challenge, including athletes, people traveling to high altitude areas, or people who live with conditions that regularly expose them to hypoxic challenge including sleep apnea, chronic obstructive pulmonary disease (COPD), high level cervical spinal cord injuries, and many others. More studies are needed to further investigate the mechanism behind these *APOE*-depended effects, thereby paving the way to discover approaches to combat deficits in respiratory plasticity.

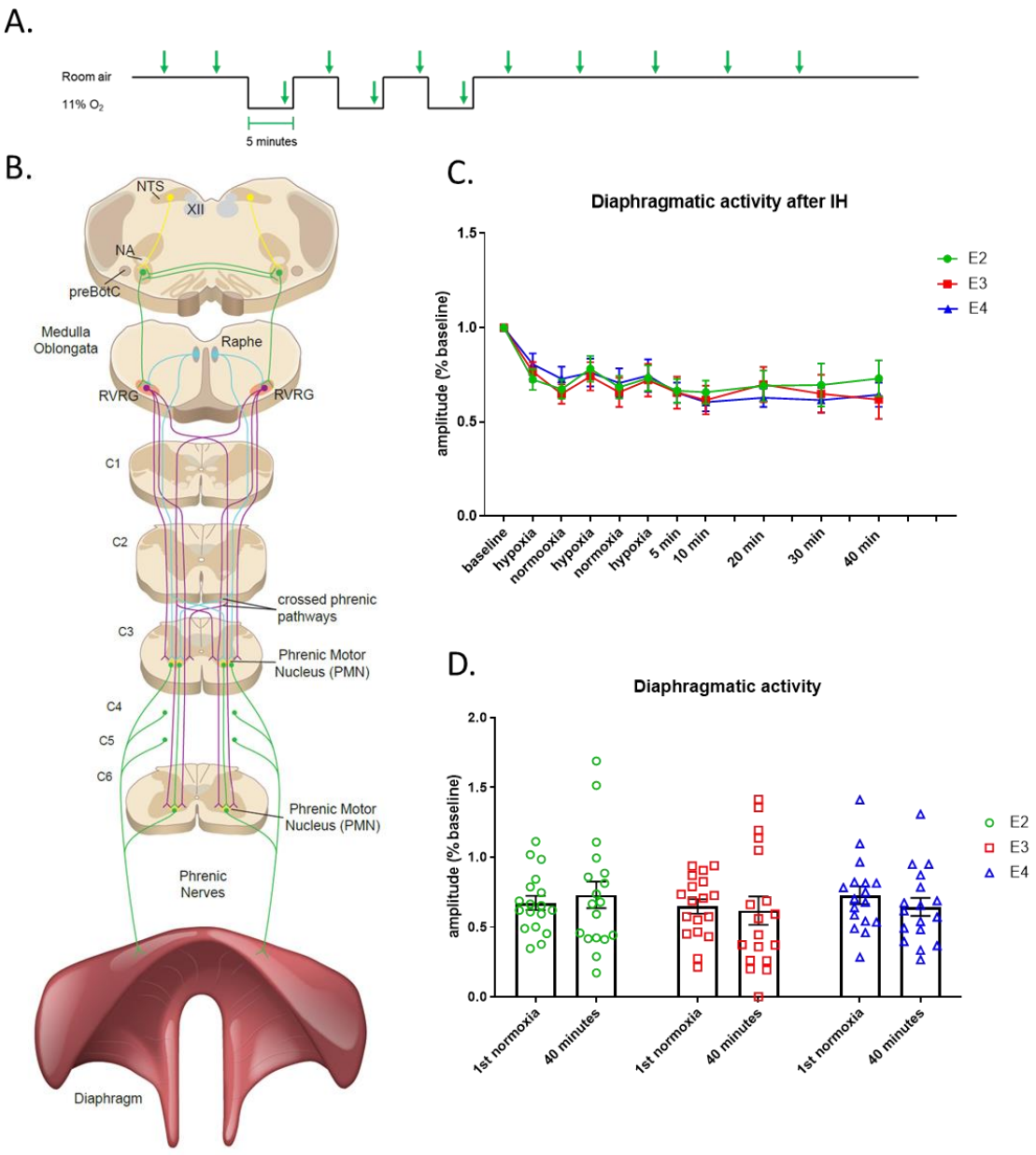

Figure 4.1 Magnitude of respiratory motor plasticity is not determined by *APOE* genotype.

A. Schematic of intermittent hypoxia protocol. Green arrows represent time point at which peak amplitude was analyzed. *B.* Schematic of the neural circuitry that mediates breathing. *C.* Amplitude of diaphragmatic bursts over time during and after IH). *D.* Comparison of burst amplitude during the first normoxic bout and 40 minutes after IH. (RMANOVA p=0.955)

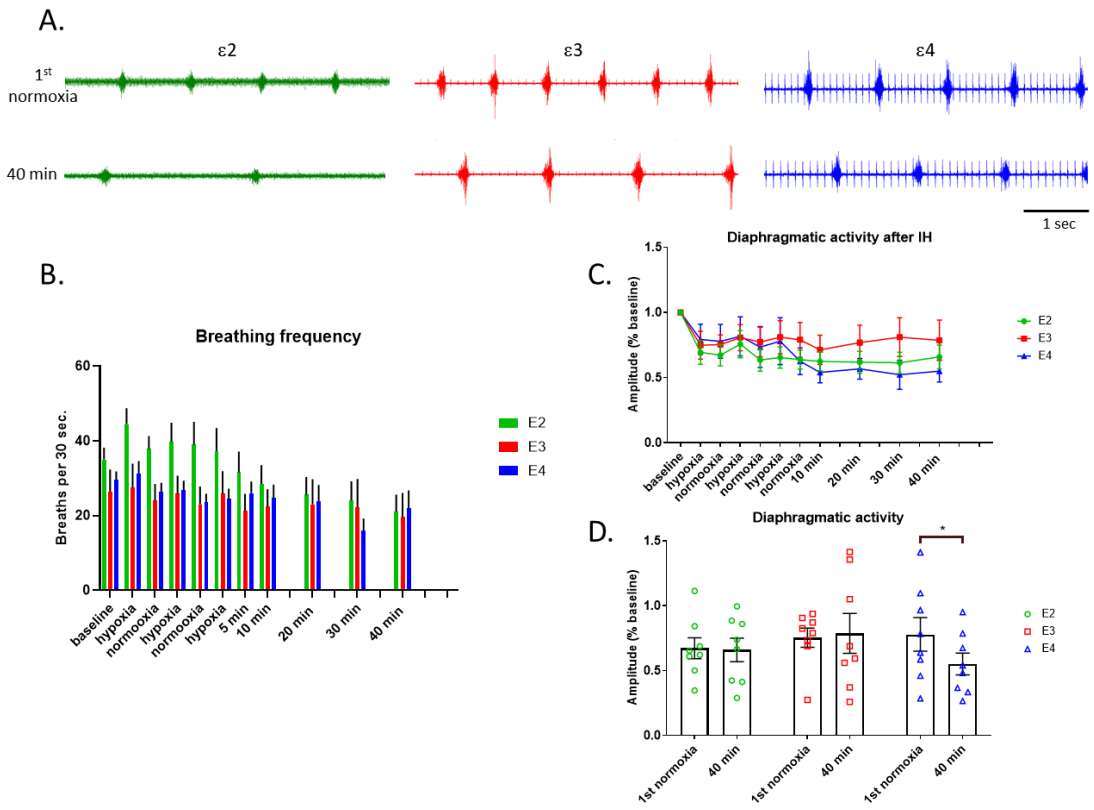

Figure 4.2 IH induces a decline in breathing activity in males expressing the human *APOE* ε4 allele.

A. Representative diaphragmatic EMG traces from E2, E3, and E4 males during the first normoxic bout and 40 minutes after IH. *B.* Breath frequency in male humanized mice decreases over time (p=0.0013), regardless of *APOE* genotype (p=0.210). *C,D.* Quantification of diaphragmatic burst amplitude during and after IH. Amplitude significantly decreases over time after IH in E4 males. (RMANOVA for a priori pairwise comparisons ε2xε2: t=0.12, ε3xε3: t=-0.29, ε4xε4: t=2.00, ε2xε3 normoxia t=-.052, ε2xε4 normoxia t=-0.68, ε3xε4 normoxia: t=-0.16, ε2xε3 40 min: t=-0.81, ε2xε4 40 min: t=0.69, ε3xε4 40 min: t=1.50).

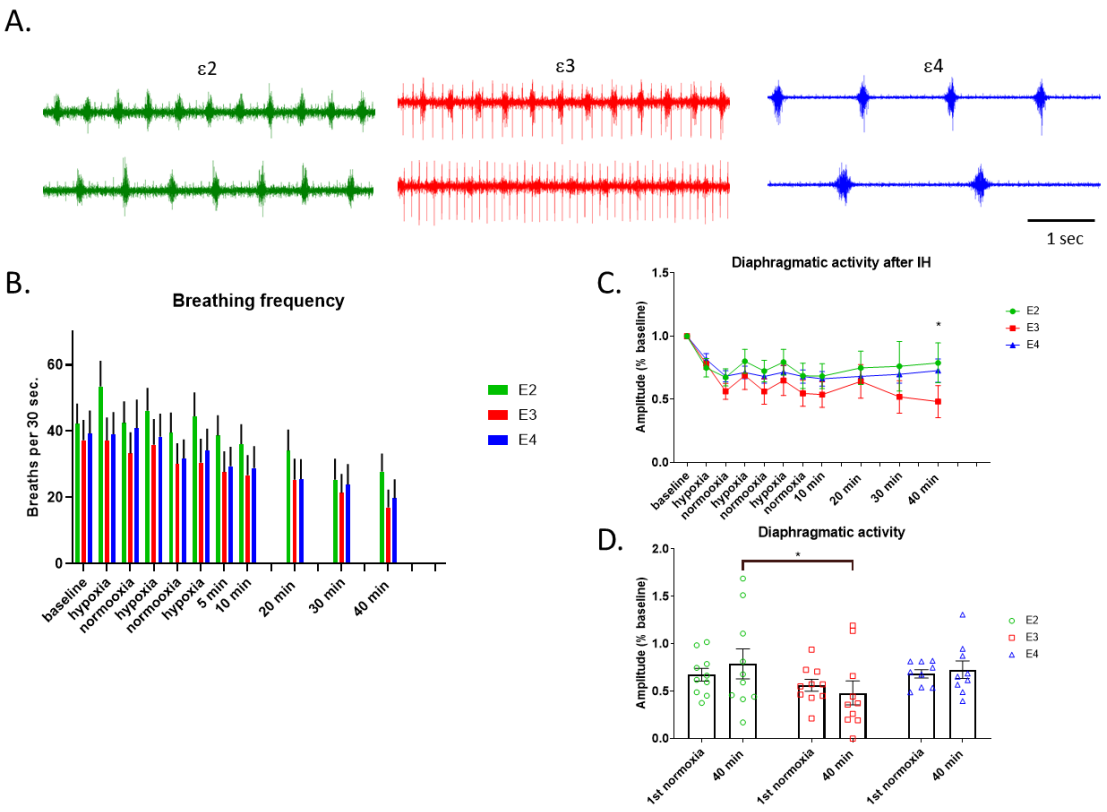

Figure 4.3 Amplitude of diaphragmatic activity decreases in response to IH in apoE3 females.

A. Representative traces from *APOE* targeted replacement mice during the first normoxic bout and 40 minutes after IH. *B.* Breathing frequency in female mice declines over time after IH ($p < 0.001$). However, there is no difference between genotypes ($p=0.429$). *C,D.* Quantification of diaphragmatic burst amplitude over time during and after IH. E3 females displayed significantly decreased amplitude at 40 minutes post IH compared to E2 females (RMANOVA with a priori pairwise comparisons ε2xε2: t=-1.05, ε3xε3: t=-1.15, ε4xε4: t=-0.39, ε2xε3 normoxia t=0.79, ε2xε4 normoxia t=-0,06, ε3xε4 normoxia: t=-0.84, ε2xε3 40 min: t=2.20, ε2xε4 40 min: t=0.43, ε3xε4 40 min: t=-1.72)

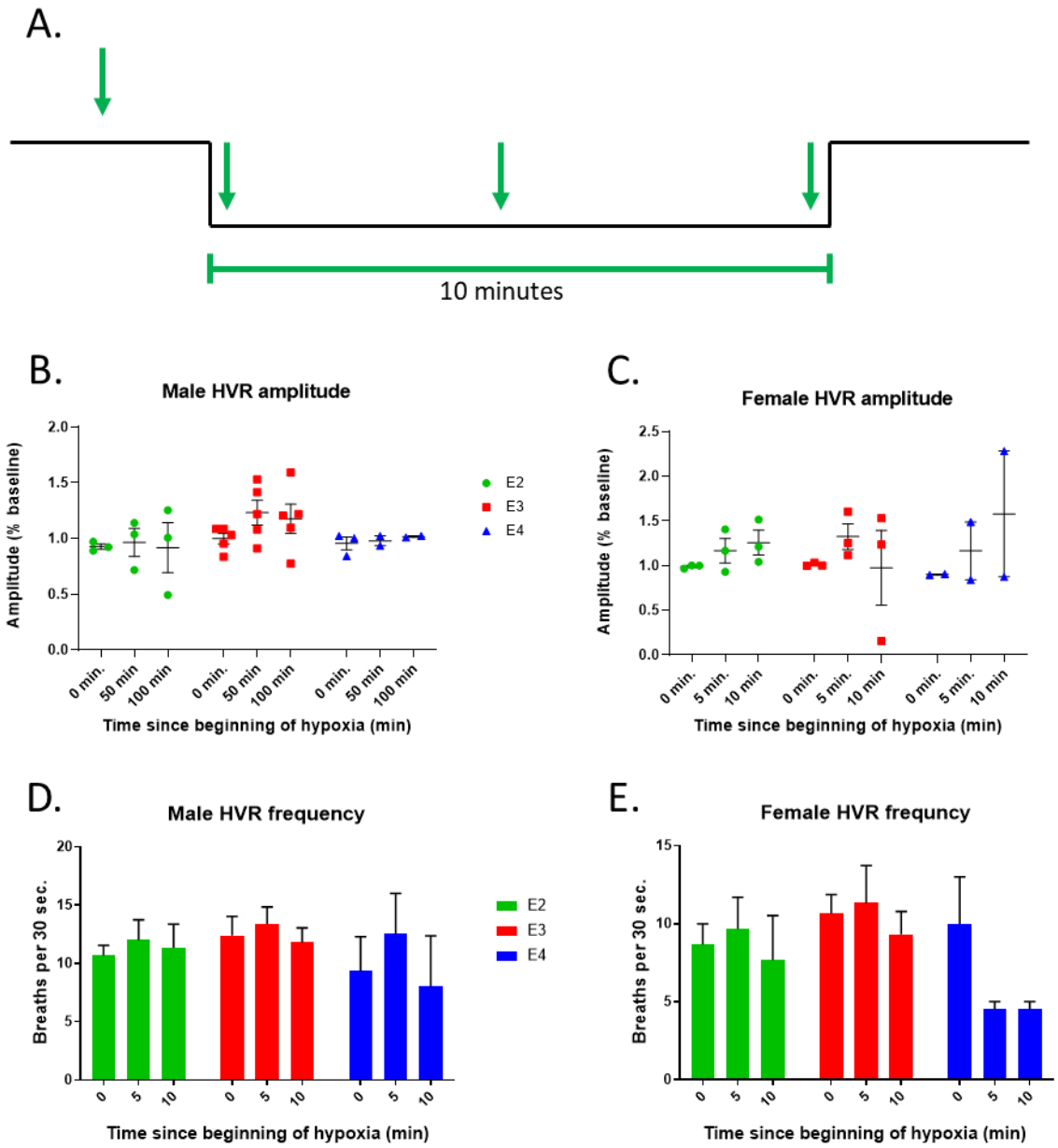

Figure 4.4 Hypoxic ventilatory response does not differ according to *APOE* genotype

A, C. Quantification of diaphragmatic burst amplitude (*A*, RMANOVA p=0.2349) and frequency (*C*, RMANOVE p=0.4756) in male mice (n=3 for each genotype). There were no statistically significant differences between genotypes. *B, D.* Quantification of the HVR response in female mice. No significant difference appears to emerge in the diaphragmatic burst amplitude (*B*). However, the frequency exhibits a decline by 5 minutes that persists at 10 minutes. No statistics were performed for female mice due to low n's (E2 n=3, E3 n=3, E4 n=2)

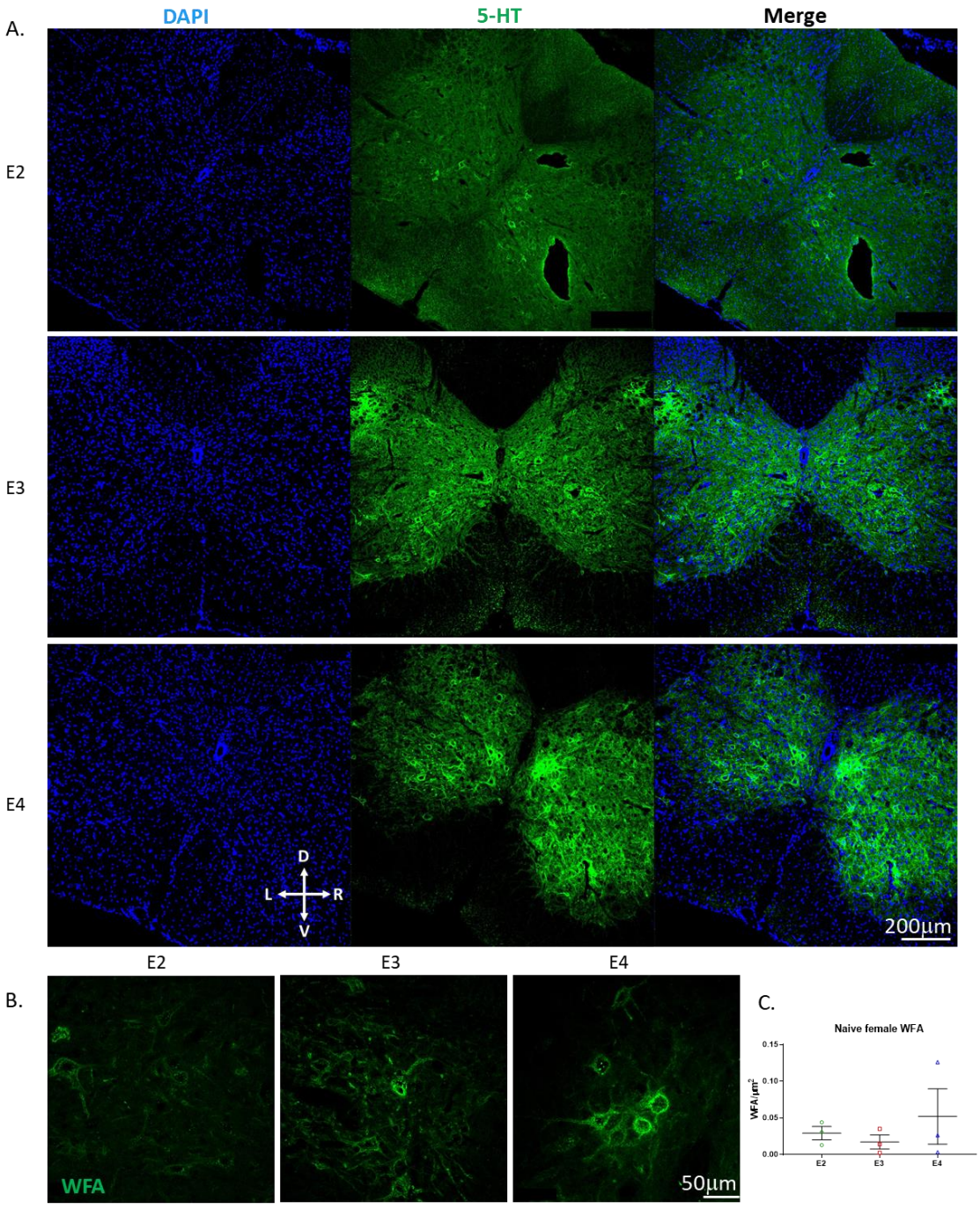

Figure 4.5 Hypoxic ventilatory response does not differ according to *APOE* genotype

A. Representative images of the spinal cord at level C4 stained for WFA, a marker of the PNN (green), and DAPI (blue). *B.* Higher magnification images of WFA staining. *C.* Quantification of WFA shows that there is no difference between genotypes (1-way ANOVA p=0.585)

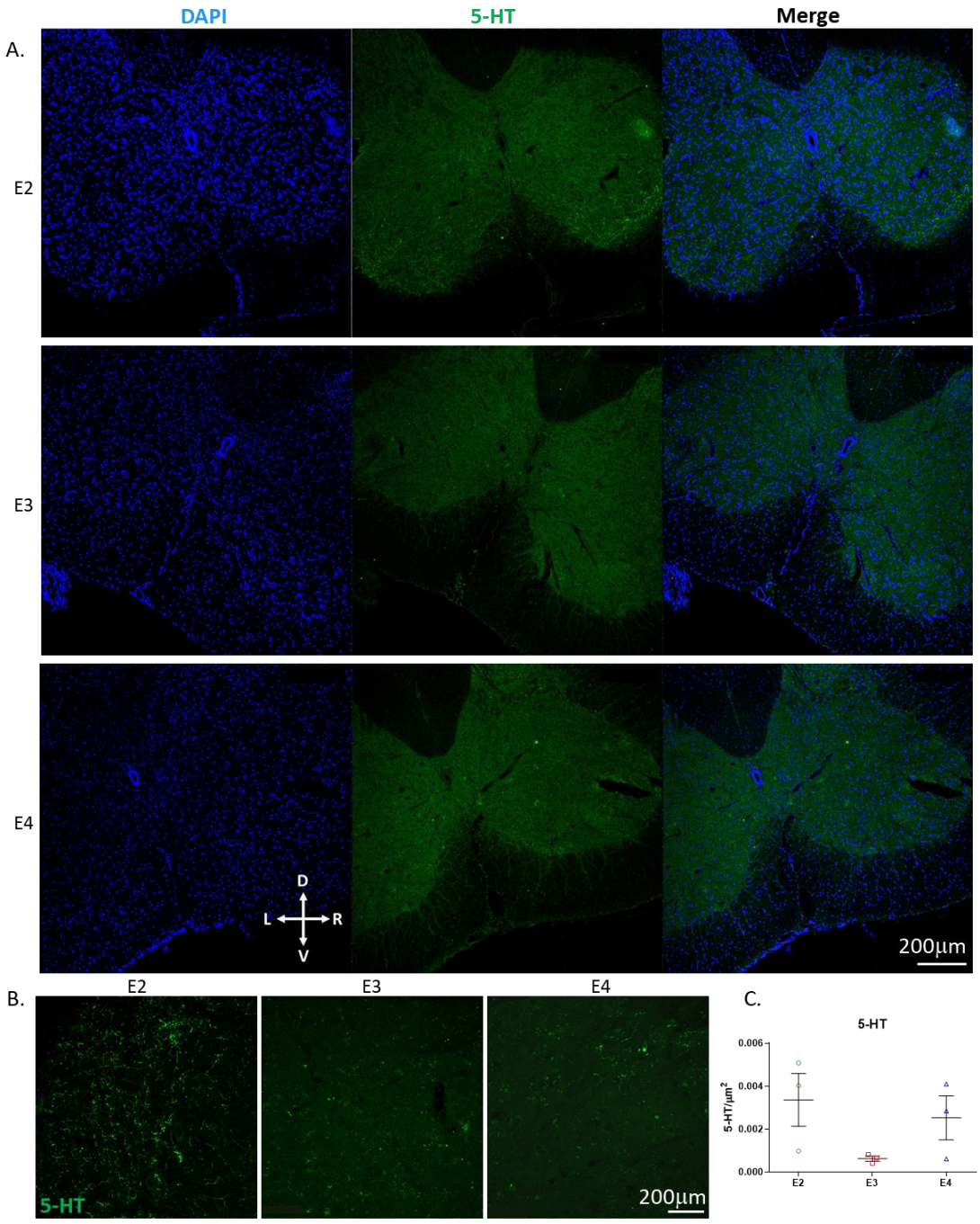

Figure 4.6 *APOE* genotype-dependent influences on 5-HT innervation of PMN.

A. Representative images of the spinal cord at the C4 level stained for 5-HT (green) and DAPI (blue). *B.* Higher magnification images of 5-HT staining. *C.* Quantification of 5-HT. There is no statistically significant difference between genotypes (1-way ANOVA p=0.182)

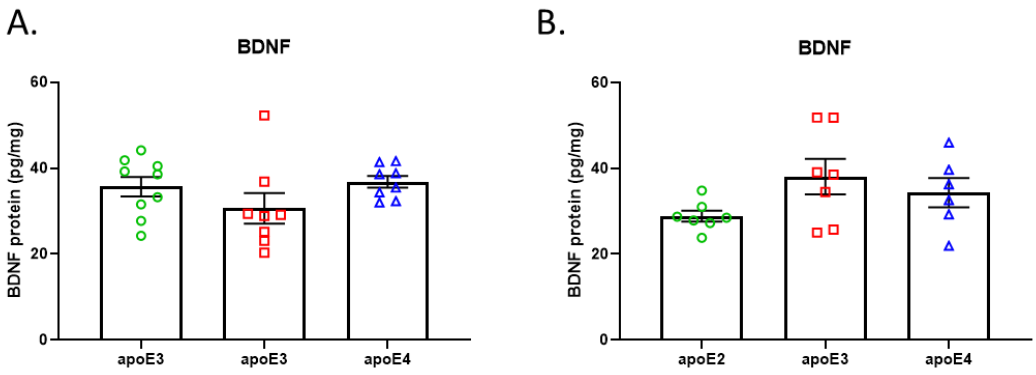

Figure 4.7 IH-induced BDNF protein synthesis is not altered according to *APOE* genotype.

Quantification of BDNF protein levels in *APOE* targeted replacement male (*A.* 1-way ANOVA p=0.216) and female (*B.* 1-way ANOVA p=0.137) mice.

CHAPTER 5. DISCUSSION

5.1 *APOE* genotype and respiratory motor plasticity

Because survival is dependent upon breathing function, the neural control of breathing must be able to adapt and maintain oxygen homeostasis in response to changes in the external environment or fluctuations in activity level and O_2 consumption. Although some of these ventilatory modifications endure only as long as the stimulus is present, others last long after the stimulus is removed. These long-lasting changes, comparable to a sort of "memory", are collectively known as respiratory motor plasticity.[507] These forms of plasticity can manifest naturally over time, as observed throughout development, or in response to injury or disease.[420,508,509] Alternatively, respiratory motor plasticity can be induced through a variety of experimental or therapeutic approaches, many of which rely on a mechanism of increased 5-HT signaling in the spinal cord.[224,510,511] While respiratory plasticity is important for maintaining O_2 homeostasis in healthy individuals, its potential to compensate for or even restore lost breathing function has even greater implications for people whose breathing circuitry has been damaged. Therefore, it is crucial that we understand how factors in the human population, such as sex, genetic make-up, and age, may positively or negatively impact individuals' ability to express this plasticity.

5.1.1 Respiratory Response to IH in the Presence of Human ApoE

The human population consists of individuals who are genetically dissimilar and it is likely that this heterogeneity results in varying aptitudes for expressing respiratory

plasticity. Therefore, to investigate the role of genotype in modulating the propensity for plasticity, we examined the impact of human apolipoprotein E (apoE) isoforms on a well-characterized model of respiratory motor plasticity. Although polymorphisms in a variety of human genes could alter spinal plasticity, a few of which were discussed in Chapter 1.3, the three alleles of the *APOE* gene were of particular interest due to the vast body of literature describing their influence on brain plasticity in health and disease, which starkly contrasts with the lack of knowledge regarding their impact in the spinal cord. Despite the relative paucity of information on apoE's role in spinal plasticity, previous work has shown that individuals who have experienced a spinal cord injury (SCI) demonstrate less motor recovery when at least one ε4 allele is present.[348,349] This suggests that *APOE* genotype does influence the plasticity of spinal circuits after injury. The current work aimed to determine if this effect was also present in the neural control of breathing and whether it was a result of spinal cord insult or if it could be observed in the intact state as well.

As a model of respiratory motor plasticity, we utilized long term facilitation (LTF), which is characterized by a measurable and prolonged increase in breathing motor output.[224,507] LTF was first discovered as a response to repeated stimulation of the carotid sinus nerve.[386] Subsequently, numerous studies in rats have investigated the signaling mechanism in phrenic motor neurons (PMNs) that leads to the augmentation of breathing activity seen during LTF, finding that it could also be induced through exposure to intermittent hypoxia (IH) or episodic intrathecal 5-HT dosing.[224,268,382,387] By applying the episodic 5-HT dosing protocol described by MacFarlane and Mitchell (2009)[382] to rats that had human apoE3 or E4 protein present in the spinal cord, we discovered that the

respiratory response to IH was not altered by the variant forms of apoE in spinally intact femaless. However, this lack of observable difference could have been due to insufficient sensitivity of diaphragmatic EMGs. It therefore did not rule out the possibility of apoE isoform-dependent influences at the synaptic level. Quantification of synaptic glutamate receptors, which are necessary for the induction of LTF[384], showed no difference between E3 and E4 animals. However, rats treated with E3 demonstrated a slight increase in synaptic AMPA receptors in response to episodic 5-HT while E4 animals did not. Although the difference in synaptic receptor levels did not reach significance, this may indicate that human E4 protein diminishes the potential for inducing synaptic plasticity in response to stimuli.

To determine whether isoform-dependent influences on spinal plasticity would be altered by SCI, these experiments were replicated in animals that undergone a C2 hemisection. The majority of SCIs occur at the cervical level, which contains axons from brainstem respiratory nuclei, as well as PMNs themselves.[464] Damage these bulbospinal fibers can disrupt breathing function, leading to diaphragm paralysis, ventilator dependence, and increased risk of life-threatening respiratory infections.[178,180] However, in a rat C2 hemisection model of SCI, LTF is capable of restoring breathing function to the ipsilateral paralyzed hemidiaphragm.[139,247] Following SCI in our animals, a trend emerged in which E3-treated rats demonstrated the expected 5-HT-induced augmentation of breathing activity while those receiving E4 did not. This trend did not reach significance, possibly due to small group sizes or inadequate sensitivity of diaphragmatic EMGs. Perhaps more insightful were the changes observed at the synaptic level. Episodic 5-HT dosing led to a significant decline in the number of synaptic NMDA receptors

localized to PMNs in spinal cords that had been treated with human apoE4. Since signaling through NMDA receptors is necessary for LTF induction, this synaptic weakening could lead to a deficits in spinal plasticity and functional recovery after SCI in individuals that express the ε4 allele.[385]

5.1.2 Interactions of Sex and *APOE* Genotype in Modulating Respiratory Plasticity

Initial studies utilizing rats treated with exogenous human apoE protein were valuable due to the amount of available literature describing the induction and manifestation of LTF in that model, which simplified experimental design and the interpretation of results. However, *APOE* targeted replacement mice that endogenously express the human apoE isoforms at physiological levels provide a more clinically relevant model. Therefore, subsequent studies examined allele-dependent influences on the propensity for LTF in humanized *APOE* mice. To explore the mechanism behind the lack of synaptic strengthening demonstrated by apoE4-treated rats, we also quantified signaling molecules that are required for LTF upstream of glutamate receptor trafficking in the mouse model.

The few studies that have characterized LTF in mice have induced LTF through exposure to IH instead of the episodic 5-HT dosing previously described in rats.[231–234] Therefore, we utilized IH as a means of inducing LTF in our humanized mice. Comparison of diaphragmatic EMG activity in hemisected *APOE* mice that homozygously express the ε3 or ε4 allele showed no allele-dependent differences in the respiratory response to IH. However, because sex is an important biological variable that modulates many processes that are relevant to the current work, such as CNS plasticity,

breathing function, *APOE* allele effects, and individuals' response to therapeutics, we also independently analyzed data from males and females.[355,358,362,442,512] There was no significant difference in diaphragmatic activity between E3 and E4 males, although IH appeared to decrease burst amplitude in those expressing ε3. This data contradicts the trend observed in the brain, where the ε4 allele is typically associated with attenuated plastic responses. This indicates that allele-dependent outcomes could be region-specific in the CNS, further emphasizing the need for deeper understanding of the influence of the unique apoE isoforms in the spinal cord. In contrast to the detrimental impact of ε3 observed in injured males, it was the ε4 allele that correlated with a significant decline in diaphragmatic burst amplitude in females. These results are in alignment with previous studies in the Alzheimer's literature reporting that detrimental effects of apoE4 are aggravated in females.[336,442]

LTF is a serotonin-dependent form of plasticity.[224] Manifestation of LTF after SCI is dependent upon the sprouting of 5-HT fibers in the spinal cord, suggesting that barriers to sprouting or regeneration would limit the capacity for respiratory motor plasticity.[247] One notable barrier to regeneration and sprouting is the presence of CSPG (chondroitin sulfate proteoglycan) molecules. The secretion of these molecules is upregulated in response to SCI, although prior to the current study, it remained unknown whether the magnitude of this upregulated is modulated by human *APOE* alleles.[99,409,422] Despite increased levels of CSPGs located around the PMNs of E4 females, this did not correspond to a deficiency in 5-HT in the same region. Indeed, 5-HT levels were significantly increased in E4 animals, compared to those expressing the ε3 allele. Since CSPGs are known to impede growth of 5-HT fibers after SCI, and because E4 females

also demonstrate elevated 5-HT levels in the intact state, this may be the result of a compensatory mechanism that maintains PMN excitability in the presence of apoE4, which is associated with loss of synaptic integrity and impaired excitatory synaptic signaling.[209,218,407,422,513] Although the availability of 5-HT in the phrenic motor nucleus may not cause the abatement of respiratory plasticity in E4 females, further studies are needed to determine whether 5-HT receptor expression and synaptic localization are altered by apoE4.

Activation of the chemosensitive carotid bodies during a single bout of hypoxia stimulates the hypoxic ventilatory response (HVR), characterized by a transient increase in respiratory output.[430] Interestingly, mice utilized in the current study displayed a decrease in breathing activity in response to a single 10 minute bout of hypoxia, as well as during the first 5 minute hypoxic bout during IH. Intrigued by this unexpected result, as well as the lack of IH-induced ventilatory augmentation that we observed in uninjured apoE-treated rats, we next investigated the respiratory response to IH in spinally intact *APOE* mice. Similar to hemisected mice, there was no difference between genotypes when males and females were grouped together. However, upon separating the sexes for further analysis, ε4 males and ε3 females displayed significant IH-induced reductions in breathing activity, opposite of the trends observed after IH in injured mice. Quantification of CSPGs and 5-HT in intact mice revealed that E4 was again associated with higher levels of both molecules when compared to E3, although this did not reach significance. As part of the signaling cascade that leads to LTF, 5-HT signaling stimulates synthesis of new BDNF in PMNs, which is necessary for IH-induced augmentation of ventilation.[268] Therefore, we also measured BDNF mRNA and protein levels in the region of the

phrenic motor nucleus, which revealed no genotype-dependent differences, indicating that apoE isoform-dependent influences on LTF signaling occur downstream of BDNF synthesis.

5.1.3 Significance of Findings

Together, results from our studies in two rodent species reveal that both sex and *APOE* genotype modulate spinal and respiratory motor plasticity. While no *APOE* genotype-dependent differences are evident when males and females are grouped for analysis, the ε3 and ε4 allele are each associated with IH-induced decline in diaphragmatic activity, depending on which sex is being evaluated. Interestingly, these effects also seem to change in response to SCI (illustrated in Figures 5.1 and 5.2). In the presence or absence of injury, LTF is induced through a mechanism of increased 5-HT signaling, which strengthens synaptic connections on PMNs by stimulating BDNF synthesis to activate downstream phosphorylation and trafficking of glutamate receptors.[224,268,288] Presence of 5-HT around putative PMN and synthesis of BDNF were not dependent on *APOE* genotype. However, levels of synaptic NMDA receptors were decreased in the presence of E4, suggesting a deficit in the signaling pathway downstream of BDNF synthesis that prevents NMDA receptor trafficking. This deficiency perhaps lies in BDNF activation of the TrkB receptor or in TrkB's kinase activity, although more in-depth mechanistic studies are needed to determine how the human isoforms of apoE interact with this signaling cascade. Primarily, our data emphasizes the importance of understanding how factors that contribute to diversity in the human population, but are often incompletely represented by preclinical animal models, can modify the efficacy of therapeutic approaches in spinally injured individuals.

This is especially crucial in the context of therapeutic IH, which has undergone clinical trials to evaluate its efficacy in enhancing breathing recovery, as well as for a variety of other functional outcomes after SCI.[41,140,142,403,404] By recognizing how factors such as sex and genetic background impact the response to therapeutics, we can begin to develop personalized approaches to overcome innate barriers to plasticity, regeneration, and neuroprotection, thereby facilitating translation of therapeutics and improving functional outcomes for people living with an SCI.

5.2 Methodological Considerations and Limitations

To accurately interpret our results, we must consider the limitations and potential pitfalls of our experimental approaches. Fortunately, a vast body of literature describing protocols of IH and other approaches to induce LTF, as well as the signaling required for its manifestation in spinally intact or injured rats, is available to provide context for our data. However, the same is not true regarding respiratory plasticity in mice. While this presented challenges for both experimental design and data analysis, it also enhances the value of the current studies for the field of SCI and neural plasticity. Examining the respiratory response to IH in both sexes of a humanized mouse model will pave the way for the use of more translationally relevant preclinical models. Due to the availability of transgenic mice that express a variety of human genes, they are powerful tools for exemplifying the diversity found in the human SCI population.

5.2.1 Induction of LTF in Rats Treated with Human ApoE Protein

Although LTF has been well-characterized as an augmentation of breathing activity in response to IH or episodic 5-HT dosing, rats in the current study did not

exhibit the expected increases in ventilation.[139,224,268,382] In the absence of injury, diaphragmatic activity remained constant in response to episodic 5-HT. Following C2 hemisection, E3-treated animals did exhibit a slight increase in diaphragmatic activity, but this change did not reach significance. Although we followed the 5-HT dosing protocol that MacFarlane and Mitchell (2009) showed to be effective in rats, some aspects of our experimental preparation differed from those previously described. Notably, many studies record phrenic neurograms instead of diaphragmatic EMGs.[247,268,382] This technique requires a phrenicotomy at least unilaterally, which removes phrenic afferent feedback and alters PMN excitability.[514,515] Indeed, rats that undergo phrenicotomy prior to IH display enhanced LTF compared to those with intact phrenic nerves.[514] Therefore the presence of intact phrenic nerves in our animals may have prevented more dramatic alterations in activity.

Many LTF preparations also include bilateral vagotomy and artificial ventilation of animals in order to gain control of blood gases without interference from lung stretch receptor feedback.[247,268,516] Because our apoE-treated rats were freely breathing, both phrenic and vagal feedback could have masked IH-induced breathing changes. An additional consequence of performing diaphragmatic EMG recordings in freely breathing animals without pCO_2 monitoring is that we could not control for changes in metabolic rate. Small mammals such as rats and mice can respond to hypoxic exposures by activating hypoxic hypometabolism, a decrease in metabolic rate which decreases O_2 consumption to negate the need for increased ventilation when exposed to low environmental O_2.[441] Due to confounding factors that may have decreased our sensitivity for detecting LTF, it is even more remarkable that we observed a divergence in the

breathing response to IH in injured E3 and E4 rats. Although the observed trend did not reach statistical significance, we did observe significant E4-dependent changes in synaptic NMDA receptor levels, indicating that human apoE4 may prevent neural plasticity and synaptic strengthening in the injured spinal cord.[384,385]

Inherent in our dosing protocol is some uncertainty regarding the diffusion pattern of intrathecal 5-HT and apoE, as well as what cell types these molecules interacted with. This 5-HT dosing protocol has been shown to induce LTF of phrenic nerve activity without altering activity of the hypoglossal nerve, suggesting that 5-HT reaches PMN in the cervical ventral horn but does not communicate with brainstem respiratory nuclei.[382] Therefore, human apoE proteins, which were applied to the spinal cord in the same manner presumably followed a similar diffusion pattern. However, we did not assess the distribution of apoE in the spinal cord or brainstem. ApoE, especially in its lipidated form binds receptors in the LDLR family, which are expressed by a variety of cell types (neurons, microglia, astrocytes, and oligodendrocytes), to activate an assortment of downstream signaling pathways.[517–520] Intrathecal administration of apoE could have led to inconsistent distribution of the protein between cell types, as well as differences between animals, increasing variability in our data. To address this uncertainty and to ensure that human apoE isoforms were present at physiologically relevant levels in the spinal cord throughout developmental stages and recovery after SCI, subsequent studies in *APOE* targeted replacement mice were crucial.

5.2.2 Insights into a Novel Approach for Studying Respiratory Motor Plasticity in Mice

The literature describing the respiratory response to IH in mice is sparse. Consequently, there is a lack of knowledge regarding best practices for induction and measurement of LTF on this model. Although a few studies have shown that mice do express LTF, the methods of induction, as well as the type of LTF evaluated (e.g. hypoglossal, ventilatory, etc.) are inconsistent. None of them utilized spinally injured animals.[231–233] Therefore, the optimal IH protocol for injured animals was determined based on previous studies in rats and mice, as well as our own observations in mice. Previous studies have shown that spontaneous recovery of diaphragmatic activity occurs by 2 weeks after lateral C2 hemisection in 40% of mice.[287] All but two animals in this study recovered EMG activity by three weeks post injury (94% had spontaneous recovery), indicating that the mice had viable synaptic inputs on phrenic motor neurons that could undergo synaptic strengthening in response to IH. In rats, which recover diaphragmatic activity much more slowly after hemisection when compared to mice (Warren et al. 2018, personal observation), the crossed phrenic pathway (CPP) is not capable of mediating IH-induced LTF for over 2 weeks post injury due to lack of 5-HT in the phrenic motor nucleus. However, Michel-Flutot et al. (2021) recently shown that the mouse CPP is effective in response to respiratory challenge such as mild transient asphyxia as early as seven days post injury[521]. Based on data from previous studies, three weeks post injury was determined to be the time point when mice would be capable of inducing LTF through synaptic strengthening of CPP inputs on phrenic motor neurons, although further studies are needed to determine how expression of mouse LTF may be enhanced or depressed at more chronic timepoints after injury.

In addition to the timing of therapeutic IH after injury, we also needed to determine the optimal IH protocol for inducing LTF. While much is known about impact of hypoxia severity and length on spinal plasticity in rats, this has not been well-described in mice.[230] Considering previous IH protocols that have been effective in mice, we chose a protocol similar to that used in freely breathing mice by Terada et al. (2008). The protocol in the current study applied three 5-minute hypoxic (11% O_2) bouts separated by normoxic periods of 5-minute duration. This was sufficient to induce increases in breathing when preliminarily tested in wild type mice (data not shown). This protocol also replicates the "modest" IH exposures commonly applied to rats in order to activate the Q pathway to LTF without increasing blood pressure, systemic inflammation, and oxidative stress, which can result from more "severe" IH.[224,268,522,523] The Q pathway is initiated through IH-induced release of 5-HT from spinal projections of the raphe.[465] This stimulates 5-HT_2 receptor signaling on PMNs and results in synthesis of new BDNF that signals through the TrkB receptor.[226,268,288,524] In contrast, more severe or sustained exposure to IH induces LTF through a 5-HT_7 or adenosine 2a receptor-dependent signaling cascade that activates PKA to stimulate synthesis and transactivation of TrkB.[477,525,526]

Both the Q and S pathway are thought to activate phosphorylation and trafficking of glutamate receptors downstream of TrkB activation, thereby strengthening synaptic connections on PMN.[226] However, simultaneous activation of Q and S cascades leads to crosstalk inhibition, preventing augmentation of breathing activity.[229,479,480] Because LTF signaling mechanisms have not been so thoroughly scrutinized in mice, it is unknown whether signaling pathways and crosstalk events are comparable across species.

Therefore, we are uncertain whether the IH protocol utilized in our study activated the Q pathway, S pathway, or both. Further studies examining how different IH strategies alter signaling in mouse PMNs will allow for optimization of IH protocols in mice, thereby ensuring that results are not confounded by crosstalk inhibition.

Another factor to be considered in the interpretation of our data and in future studies is the influence of circadian rhythms on breathing control and plasticity. Humans and rodents exhibit oscillations in body temperature, metabolism, and ventilation throughout a 24-hour period.[527] Diaphragmatic EMG recordings in our rats and mice were performed at varying times throughout the day and therefore animals were not consistently in the same part of the circadian cycle. In addition to potential impacts on baseline respiratory activity in these animals, the circadian clock also regulates various types of neuroplasticity, including respiratory motor plasticity in the form of LTF.[528–530] Activity of the serotonergic raphe exhibits rhythmicity with the sleep/wake cycle and PMNs themselves express circadian clock genes.[530,531] Expression of 5-HT_2 receptors, BDNF, and TrkB also changes throughout the day, suggesting that animals exposed to IH or episodic 5-HT at different times may have varied in their propensity for plasticity.[530] Although disregarding time of day as a biological variable may have dampened the treatment effect in our animals, it simultaneously increases clinical relevance because studies investigating the neuroplastic response to IH in spinally injured individuals have also not performed IH at the same time of day in every subject.[41,142,381,403,404]

Similar to influences of circadian rhythms, hormone cycles could also impact the propensity for plasticity in our animals. The estrous cycles of the female rats and mice in the current studies were not tracked to determine levels of progesterone or estrogen at the

time of treatment or diaphragmatic EMG recording. Progesterone and estrogen receptors are present in the CNS, including in the region of raphe serotonergic neurons. Here, signaling of these hormones increases expression of tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis.[532] Estrogen has also been shown to enhance levels of 5-HT_2 receptors and progesterone functions as a breathing stimulant that has been used to treat respiratory disorders.[374,533,534] Interestingly, LTF in males requires conversion of testosterone to estradiol and female LTF is more pronounced during stages of the estrus cycle when 5-HT levels are high, emphasizing the importance of sex steroid hormones in 5-HT-depended spinal plasticity.[372,373,535] Indeed, Dougherty et al. (2017) found that induction of LTF is enhanced when circulating estradiol levels are highest. After ovariectomy-induced abolishment of LTF, administration of exogenous estradiol is sufficient to restore respiratory motor plasticity.[536] Because we did not control for estrous cycle phase in our female animals, differences in hormone and 5-HT levels may have increases inter-animal variability and limited our ability to detect apoE isoform-dependent effects.

Measuring the respiratory response to episodic 5-HT dosing and IH without controlling for time of day and levels of sex hormones may contribute to variability in our data and limit the interpretation thereof. However, this paradigm more accurately reflects the condition of human subjects receiving therapeutic IH in clinical investigations where conditions cannot be so tightly controlled. A limitation for the translational applicability of the current studies is that homogenous animal populations and preclinical injury models are not representative of human SCI. While the lateral C2 hemisection is a reproducible injury that ensures the sparing of crossed phrenic fibers, it does not

accurately model the diversity of SCIs seen in people. Human injuries are more closely replicated by contusive injury models. While less reproducible, contusion injuries realistically injure a variety of spinal fibers, including those of the CPP, which are likely to be damaged in individuals who have experienced a cervical SCI. Unfortunately, this means that C2 contusive injury models, unlike C2 hemisections, can lead to complete loss of breathing function in animals, presenting ethical and logistical concerns. To most accurately examine the potential for plasticity and recovery of function after cervical SCI, previous work has shown the viability of contusing the cord caudal to our hemisection injury at the C3/C4 level to preserve independent ventilation.[210,537,538] Performing a similar contusive injury in *APOE* mice would strengthen the clinical relevance of our studies, an effort that is crucial to improving translation of therapeutic approaches for SCI. Indeed, a recent review by Fouad et al. (2020) emphasizes the importance of incorporating factors which cannot be controlled in the human population (sex, age, genetics, injury pathology, etc,) into preclinical studies to increase the validity of animal models, despite the risk of contributing to data variability.[377]

5.3 Future Directions

While the above data provides evidence that both *APOE* genotype and sex influence spinally mediated plasticity in rodent models of LTF, our results have also generated many more questions regarding the mechanisms behind these changes. Of particular interest is our description of how humanized mice respond to IH, which differs from the ventilatory response in rats. Future studies will contribute to our understanding of the role of apoE isoforms in modulating the function and plasticity of neural circuitry, including its interactions with various cell types in the spinal cord. Crucially, based on

our findings, future investigations will examine ways to address apoE isoform-dependent deficiencies in recovery of function and the response to SCI therapeutics. This will enhance the potential for translation of therapeutic approaches that will better serve the spinal cord injured population.

5.3.1 ApoE-astrocyte interactions in spinal and respiratory plasticity.

Astrocytes are the primary producers of apoE in the central nervous system.[303] ApoE that is synthesized and lipidated by astrocytes is internalized by neurons upon receptor binding, thereby providing cholesterol for growth and synaptogenesis.[308] While this uptake of apoE is crucial for the modulation of neuronal activity, apoE also impacts the functioning of astrocytes themselves. During development, these cells play an important role in synapse remodeling. Each astrocyte can ensheath thousands of synapses and is sensitive to synaptic activity. In response to prolonged inactivity of excitatory or inhibitory synapses, astrocytes can phagocytose and degrade synaptic material to refine developing neural circuitry.[323,539] Interestingly, during the first step of this process, the function of phagocytic receptors are modulated by ABCA1, the transporter responsible for astrocytic lipidation of apoE.[540] The three isoforms of apoE uniquely interact with ABCA1, leading to different lipidation profiles of brain apoE.[395,397] In addition to transporting lipids extracellularly, ABCA1 also translocates lipids within the membrane, suggesting that the apoE isoforms may alter how ABCA1 controls local membrane phospholipid composition and phagocytic receptor localization.[540] During development, this could lead to *APOE* allele-dependent differences in synaptic connectivity in the breathing circuitry. Specifically, apoE4 impairs phagocytosis, which could explain the

increased 5-HT+ fiber staining in the phrenic motor nucleus of ε4 mice in the current study (Chapter 3&4).[324] Future studies investigating synaptic pruning mediated by in spinal cord astrocytes from *APOE* targeted replacement mice would provide further insights into the development of breathing circuitry and PMN synaptic connectivity in intact, genetically distinct spinal cords.

Following SCI, phagocytosis is crucial to remove debris created by injury-induced Wallerian degeneration, apoptosis, and demyelination. In the presence of apoE4, reduced phagocytosis could allow lingering breakdown products to activate persistent immune responses, aggravating secondary injury cascades and potentially leading to auto-immunity in the CNS.[541,542] Investigating phagocytic processes in spinal astrocytes could reveal whether the *APOE* alleles alter the ability to remove inflammatory mediators from the injury milieu in order to prevent tissue damage. Genotype-dependent differences in this process could perhaps contribute to the neuroanatomical-functional paradox of SCI described by Fouad et al. (2020).

In addition to phagocytic processes, the apoE isoforms also modulate astrocytic metabolism. Astrocytes are not only important for exporting lipids for apoE-mediated transport, they are also the primary CNS cell type performing β-oxidation of fatty acids.[543] Indeed, cortical astrocytes contain higher levels of tricarboxylic acid (TCA) cycle enzymes than neurons, contributing significantly to the 20% of energy produced in the brain that is produced by fatty acid oxidation.[501,544,545] The fatty acid octanoate easily crosses the blood brain barrier and is responsible for much of the brain's anaplerotic flux, contributes to gluconeogenesis for glucose oxidation, and itself undergoes fatty acid oxidation to provide energy.[544] Due to the importance of metabolism of lipids such as

octanoate as precursors to ATP synthesis in the CNS, *APOE* genotype-dependent effects on lipid homeostasis have been of interest in the Alzheimer's Disease literature. Human subjects expressing the ε4 allele perform β-oxidation at a higher rate than those who lack ε4.[546,547] This hyperactivity and increased ATP synthesis is associated with cognitive impairment, an effect aggravated by age.[548,549] However, some studies also indicate that the apoE4 protein leads to mitochondrial dysfunction and reduced ATP synthesis.[506,550] In addition to its influence on lipid metabolism, apoE4 has also been associated with impaired glucose uptake and hypometabolism.[502,551] Despite contradictory conclusions about the impact of apoE isoforms on ATP production, these studies demonstrate that apoE modulates flux through metabolic pathways in neurons and astrocytes.

Measuring bioenergetics of astrocytes found in the phrenic motor nucleus or brainstem respiratory nuclei before and after SCI could reveal how apoE isoforms influence metabolism to change breathing function. SCI induces to a significant loss of mitochondrial function, leading to free radical production and exacerbating secondary damage, which may be further intensified depending upon apoE isoform-dependent impacts on mitochondria in the cervical spinal cord.[11] Mitochondria could therefore be a powerful therapeutic target for alleviating isoform-dependent deficits in metabolism to enhance respiratory plasticity and the recovery of motor function after SCI.[552,553]

Astrocytes contribute to the neural control of breathing in both the intact and injured state. In the rhythmic pre-Bötzinger complex (preBötC), astrocytes contribute to central chemosensation. Ablation of brainstem astrocytes attenuates the HVR, similar to the effect of inhibiting peripheral chemosensation of the carotid body, emphasizing the importance of this central CO_2 sensitivity. Detection of elevated pCO_2 and low pH

stimulates the release of vesicular ATP.[554–556] Rhythmogenic preBötC neurons are excited by ATP and respond to purinergic signaling by increasing inspiratory frequency, thereby augmenting breathing activity upon astrocytic detection of low pH.[557,558]

At the level of the phrenic motor nucleus in the cervical spinal cord, astroglial cells interdigitate with PMN, preventing direct communication between the motor neuron dendrites.[559] Within hours after a C2 hemisection, these astrocytic processes actively retract, allowing the number of dendrodendritic appositions to increase, which is accompanied by the formation of additional excitatory bulbospinal synaptic connections on PMNs.[560,561] This is thought to be a mechanism of plasticity contributing to activation of the latent crossed phrenic pathway after cervical SCI. *APOE* genotype-dependent influences on astrocytic metabolism or Ca^{2+} homeostasis may impact their ability to undergo structural reorganization that prompts strengthening of PMN connectivity. ApoE isoforms could also alter their capacity for sensing and transmitting information about brainstem CO_2 levels through ATP release, potentially attenuating the respiratory response to hypoxia. Future studies investigating the impacts of apoE on astrocytes' chemosensation function and capacity for remodeling in the breathing circuitry would contribute to our understanding of how genetic diversity in the human population may influence individuals' capacity for respiratory plasticity in response to injury and ventilatory challenge.

5.3.2 The inflammatory milieu after SCI: impact of *APOE* genotype

As discussed in Chapter 1.1.1, the inflammatory response to SCI is a dynamic process that can paradoxically contribute to secondary injury, as well as neuroprotection

and neural plasticity. Interestingly, compared to apoE3 animals, humanized E4 mice express higher levels of pro-inflammatory cytokines after CNS insult, which is associated with a decline in synaptic markers.[562–565] Clinically, the importance of understanding how apoE isoforms modulate CNS inflammatory responses is underscored by data showing that anti-inflammatory drugs can reduce risk of Alzheimer's, but only in individuals expressing apoE4. This suggests that some detrimental effects of the E4 isoform may be alleviated by targeting neuroinflammation.[566]

In addition to *APOE* genotype-dependent influences, inflammatory profiles after neurotrauma are also modulated by factors such as age and sex. Genotype-dependent effects in our data diverged according to sex, indicating that it will be important to investigate the mechanism behind the observed sex differences. As previously discussed, LTF itself is modulated by levels of sex hormones, especially estrogens, which are required for LTF even in males.[371–373,434] Following neurotrauma, the acute inflammatory response to CNS injury also differs between males and females, with males exhibiting greater microglial activation and peripheral myeloid cell infiltration.[567,568] The divergent response of immune cells is attributed to the immunosuppressive properties of estrogens. These female sex hormones attenuate the release of pro-inflammatory cytokines, upregulate anti-inflammatory cytokines, and enhance the T-cell response to clear myelin debris and improve functional outcomes in females.[569,570] In contrast to estrogens, testosterone is associated with excitotoxicity and oxidative stress after SCI.[571,572] Sex hormones also impact mitochondrial function, as demonstrated by the role of estrogens in maintaining metabolic homeostasis.[573] Breakdown of metabolic processes after SCI further exacerbates secondary injury cascades, suggesting an additional neuroprotective

advantage provided by female sex hormones.[11,574] Conclusions from these previous studies indicate that *APOE* genotype and sex may interact to modulate the inflammatory response to SCI, leading to differences in respiratory plasticity and functional recovery.

To determine how sex and *APOE* alters respiratory and spinal plasticity through modulating inflammation, future studies will utilize a more recently characterized model of plasticity that relies on the presence of inflammatory mediators. This form of respiratory motor plasticity is known as inactivity-induced motor facilitation (iPMF) and leads to augmentation of breathing activity similar to that observed during LTF.[238] However, it differs from LTF in both its means of induction and the signaling pathways that lead to its manifestation. While LTF develops in response to heightened inspiratory drive elicited by exposure to hypoxia, iPMF results from a lack of respiratory drive. High levels of mechanical ventilation removes the drive to breath, leading to a neural apnea. When inspiratory drive is restored and phrenic nerve activity returns, compensatory plasticity becomes evident in the form of enhanced phrenic activity.[238] This facilitation is dependent upon signaling of the TNF-α cytokine, implicating the involvement of inflammatory responses to apnea.[237] Therefore, evaluating lesion characteristics and iPMF in male and female mice expressing human *APOE* alleles will provide insight into how sex and *APOE* genotype impact both immune activation and plasticity after SCI.

Astrocytes may also be key mediators in the induction of iPMF. Astrocytes are known to respond to synaptic inactivity by releasing TNF-α. This signaling stimulates synaptic localization of glutamate receptors in neurons, thereby strengthening synaptic connections.[241] Although this mechanism is typically associated with gradual changes in synaptic scaling, it could also contribute to more acute synaptic enhancements that lead to

the development of iPMF[34]. Utilizing iPMF as a model of plasticity in intact and spinally injured animals will allow for the investigation of how astrocytic activation and signaling, glutamate receptor trafficking, and respiratory plasticity are impacted by *APOE* genotype.

5.3.3 Considering age as a biological variable in studies of the apoE isoforms.

APOE rose to notoriety in the Alzheimer's Disease field as the most significant genetic risk factor for developing this disease. However, Alzheimer's is a disease of aging, and accordingly, age is the primary determinant of Alzheimer's risk. Aging further exacerbates apoE4- and sex-dependent effects on brain pathology and cognitive decline. Indeed, previous data indicates that apoE4 may enhance synaptic plasticity in some brain regions in young humanized *APOE* mice, but this effect disappears with age.[447,448] Females are at greater risk of developing Alzheimer's, as well as more pronounced E4-dependent tau pathology and deficits in neural circuitry connectivity even before disease onset.[575,576] In aged mice expressing ε4, estradiol replacement therapy enhances LTP, improves memory, and reduces Alzheimer's risk, indicating that female sex hormones are neuroprotective against detrimental effects of apoE4 in the CNS.[577,578]

Together, evidence from the Alzheimer's Disease literature underlines the importance of considering both age and sex as biological variables when investigating the influence of apoE isoforms on neural plasticity and CNS disease outcomes. This is especially relevant for the current work because spinal cord injuries are becoming increasingly common in older individuals. SCIs used to be a young person's injury, with an average age of 29 at the time of injury in the 1970's. As people live longer and the

population ages, the average age has increased, with falls emerging as a more prevalent cause of injury in the older population. Although the average age of injury is currently 43, SCIs have a bimodal distribution, proving to be most common in young adults and elderly individuals.[2,579] In our study, *APOE* humanized mice were 3 months old (representative of young adults) when they received an SCI. Future experiments using aged animals would be valuable in determining whether age exaggerates genotype and sex effects on spinal and respiratory plasticity in a manner agreeing with trends described in the neurodegeneration literature.

SCI in older individuals elicits pathological responses that are distinct from those found in young adults, activating secondary injury cascades that could determine the extent of tissue damage, injury severity, and the response to therapeutic interventions.[580–583] Clinically, it is difficult to isolate age-dependent influences on outcomes after SCI due to a variety of covariates including age-related comorbidities, lack of precise outcome measures, and variability in injury characteristics.[584] For example, lengths of stay in rehabilitation facilities are reduced in the older SCI population because older individuals are more likely to sustain incomplete lesions. Despite the higher prevalence of complete injuries, younger people still recover independent walking after SCI more frequently than elderly individuals.[585] Due to the challenging nature of clinical SCI aging studies, it is important to develop preclinical models that will accurately reflect the human condition, including representation of genetic diversity, sex, and age. By utilizing humanized mouse models, future studies will provide a means of studying how *APOE* genotype-dependent injury pathology evolves with age.

Previous studies have shown that mitochondrial function declines with age, leading to increased production of reactive oxygen species.[586] Some types of age-dependent mitochondrial dysfunction are more pronounced in the spinal cord than in the brain, suggesting that if apoE isoforms inhibit spinal plasticity through altering mitochondrial bioenergetics, these detrimental effects will be exacerbated by aging.[587] Accordingly, the E4 isoform is associated with heightened oxidative damage in the Alzheimer's brain, although E4's impact on spinal cord ROS production remains unexplored.[588] However, in wild type animals, increased mitochondrial ROS production after SCI activates spinal cord macrophages, which more commonly develop a pro-inflammatory phenotype in aged animals.[580,581] Strikingly, even M2 macrophages, which are typically considered protective in young animals, produce more ROS while concurrently releasing fewer anti-inflammatory cytokines after SCI in older animals.[580,582] Reducing macrophage activation and ROS production enhances neuroprotection and improves functional recovery after SCI in aged mice, presenting a promising therapeutic target if apoE isoform-dependent effects on oxidative stress and immune activation are discovered.[583]

5.3.4 Therapeutic interventions for overcoming apoE-induced constraints on breathing plasticity

The emergence of *APOE* genotype-dependent impacts on respiratory and spinal plasticity in our data raises a critical question: how do we ameliorate functional deficits that result from an individual's genetic make-up? While sex differences also contributed to genotype effects in humanized mice, it is possible that sex effects could be ameliorated

through hormone replacement. In the brain, estradiol replacement has shown efficacy in enhancing plasticity to improve learning and memory in women who express the ε4 allele.[577,578] Estradiol may similarly restore spinal plasticity in ε4 females, while also acting as a neuroprotective antioxidant after SCI.[589] Because previous data has also indicated that excess hormone replacement can exacerbate brain aging in females who lack the ε4 allele, the effects of estradiol on CNS function and plasticity in the presence of human *APOE* alleles must be further investigated to assess its potential risks and benefits.[590] Estrogen signaling is also important for plasticity in the male CNS, which is emphasized by data showing that estradiol is required for LTF in male rats.[372] Indeed, estradiol treatment can improve functional outcomes in male rats that sustain a traumatic brain or spinal cord injury, emphasizing the importance of testing its therapeutic potential in both sexes of humanized *APOE* mice.[370,591,592]

Although modulating the levels of sex hormones may alleviate some of the observed impacts of *APOE* genotype on the propensity for plasticity in our animals, a more effective approach may be to directly target apoE proteins. To overcome barriers to plasticity that arise from the unique structures and molecular interactions of each apoE isoform in the brain, apoE mimetic peptides and structure correctors have been developed. The amino acid substitutions at positions 112 and 158 lead to different intramolecular interactions in apoE isoforms, altering their ability to interact with other molecules.[593] Protein levels of apoE in the CNS are also *APOE* allele-dependent, with lower levels observed in individuals expressing ε4.[594] This suggests that interactions between apoE and its receptors are altered in E4 animals due to both its structure and decreased availability in the CNS.[396] Mimetic peptides are derived from the receptor

binding domain of apoE, which is common between the E2, E3, and E4 proteins. This allows the peptides to interact with apoE receptors and mediate the neuroprotective and anti-inflammatory actions of apoE3.[595–597] Indeed, mimetic peptides have shown preclinical efficacy in reducing CNS inflammation and improving functional outcomes after SCI and TBI, in addition to protecting against Alzheimer's pathology.[598,599]

Mimetic peptides increase apoE receptor activation by increasing levels of the receptor binding domain that can effectively bind apoE receptors. In contrast, small molecule structure correctors interact with the apoE4 protein itself in order to interrupt intramolecular domain interactions that result from the Cys112Arg substitution. This causes E4 to acquire a more open tertiary structure that resembles E2 or E3.[600–602] Structure correctors prevent apoE4-induced impairment of neurite outgrowth and mitochondrial dysfunction.[601,603] Although this therapeutic strategy has not been tested in the context of SCI, future exploration of the possibility of converting apoE4 into the functional equivalent of apoE3 could reveal enhanced respiratory plasticity in spinally injured E4 females.

While directly targeting the structure or molecular interactions of apoE isoforms may alleviate their detrimental impacts on CNS function and plasticity, they could also have unintended side effects in the periphery, where apoE is also highly expressed.[317] Therefore, it may be more translationally relevant to target signaling pathways that are altered by apoE isoforms in the brainstem and spinal cord where the neural circuitry that mediates breathing is housed. One crucial signaling molecule that is modulated differentially by the apoE isoforms in the CNS is calcium.[604] Specifically, apoE4 disrupts Ca^{2+} homeostasis by increasing influx of extracellular Ca^{2+}. The resulting increase in

intracellular calcium levels is associated with increased neuronal cell death.[328] SCI also stimulates Ca^{2+} influx, further exacerbating the disruption of Ca^{2+} homeostasis caused by apoE4, which could explain why detrimental effects of E4 were only observed after injury in our rat model.[605]

Mitochondria function as cellular Ca^{2+} regulators. Excessive Ca^{2+} can interrupt mitochondrial bioenergetics and lead to opening of the mitochondrial permeability transition pore, allowing for the release of reactive oxygen species and apoptotic proteins.[553,606] As previously discussed, oxidative damage stimulates inflammatory processes and vice versa, demonstrating that loss of Ca^{2+} homeostasis has significant implications for metabolism and secondary injury after SCI.

High calcium levels could also inhibit synaptic plasticity through activation of calcineurin, a Ca^{2+}-dependent phosphatase[607]. While some entry of Ca^{2+} through calcium-permeable AMPA receptors can be lead to beneficial enhancement of synaptic transmission, excessive Ca^{2+} signaling can have the opposite effect.[608] Increased Ca^{2+} influx in mice expressing ε4 is associated with heightened activity of calcineurin, which reduces the density of dendritic spines and inhibits synaptic plasticity, possibly through dephosphorylation of glutamate receptors.[329,609] Together, previous studies describing the interactions of apoE isoforms, Ca^{2+} signaling, cytotoxity, and synaptic plasticity suggest that restoring calcium homeostasis may alleviate E4-associated deficits in respiratory plasticity before or after SCI. The therapeutic efficacy of approaches that prevent mitochondrial permeability transition or which reduce calcineurin activity, such as cyclosporine A or FK-506, respectively, should also be investigated for their ability to promote neuroprotection and spinal plasticity in humanized *APOE* mice.[610–613]

5.4 Conclusion

Together, our data shows that both sex and human *APOE* genotype determine the propensity for spinal and respiratory plasticity. When considered in the context of the neurotrauma and Alzheimer's Disease literature, this general conclusion is not surprising, although the specific genotype-dependent responses did not follow the patterns that have previously been described in the brain. The ε4 allele has previously been associated with attenuated synaptic plasticity and worse functional outcomes in traumatic brain injury and Alzheimer's, effects which are more pronounced in females. However, the current study demonstrated that both the ε3 and ε4 alleles can inhibit spinal and respiratory plasticity, depending on sex and the injury status of the spinal cord. This is the first investigation of how genetic diversity in the human population may alter the propensity for spinal and respiratory plasticity, as well as the ability to respond to SCI therapeutics. Our novel findings underscore the importance of considering biological variables in the human SCI population when developing therapeutic approaches. While preclinical animal models can never comprehensively represent the extent of diversity found in people, utilizing multiple species, transgenic animals, both sexes, and clinically relevant injury models can provide better predictors of the efficacy of treatment strategies in diverse patient populations. Understanding how factors such as *APOE* genotype impact the response to therapeutics that are entering clinical trials, including IH, will aid in the development of personalized strategies to overcome barriers to functional recovery. Fundamentally, our data will pave the way for improving translation of SCI therapeutics in order to improve functional outcomes and quality of life for individuals living with SCI.

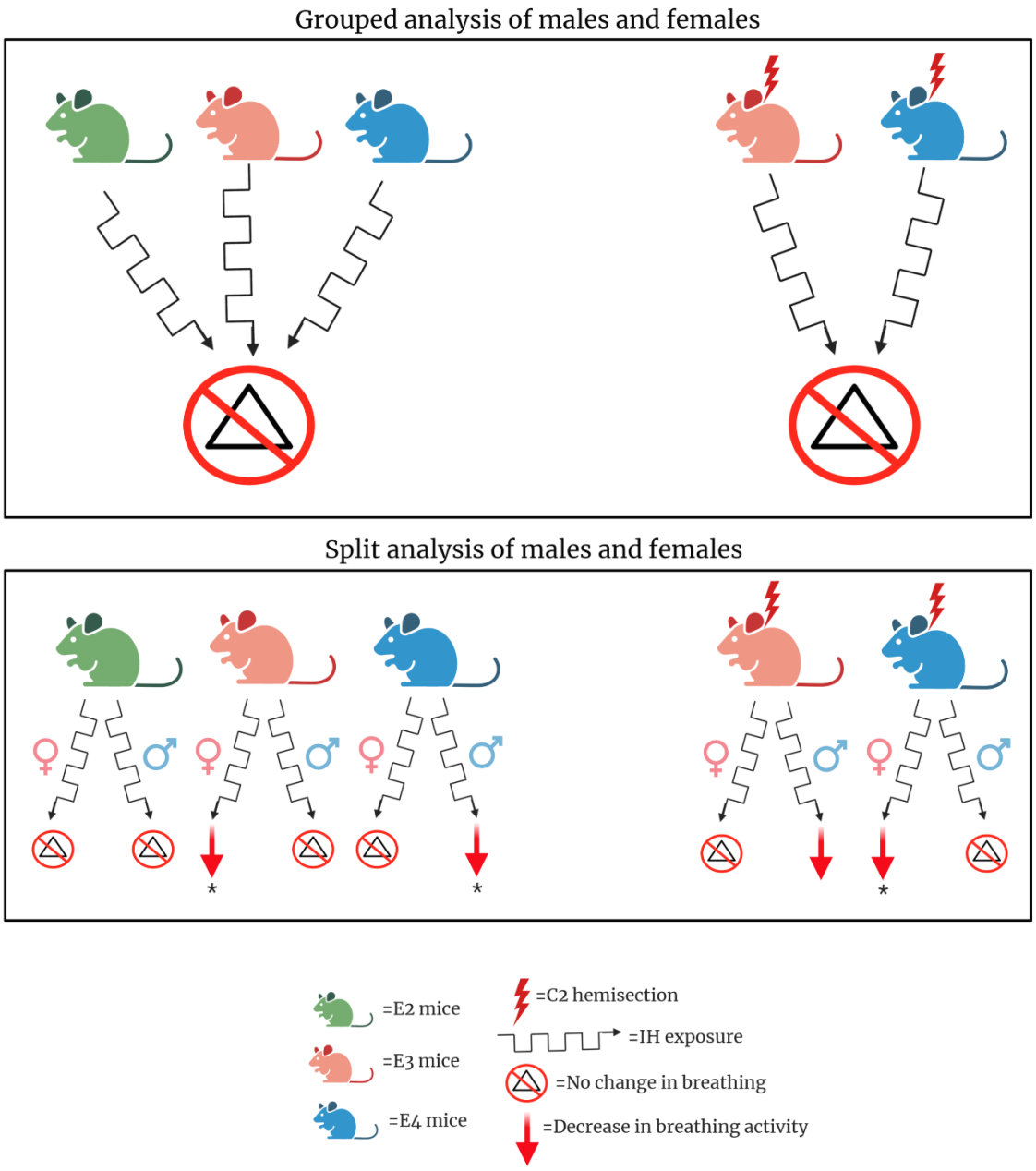

Figure 5.1 Graphic summarizing sex- and genotype-dependent differences in the respiratory response to IH

Injured (left) and C2 hemisected (right) *APOE* targeted replacement mice displayed unique responses to IH, which were dependent upon sex and *APOE* genotype. (Created with BioRender.com)

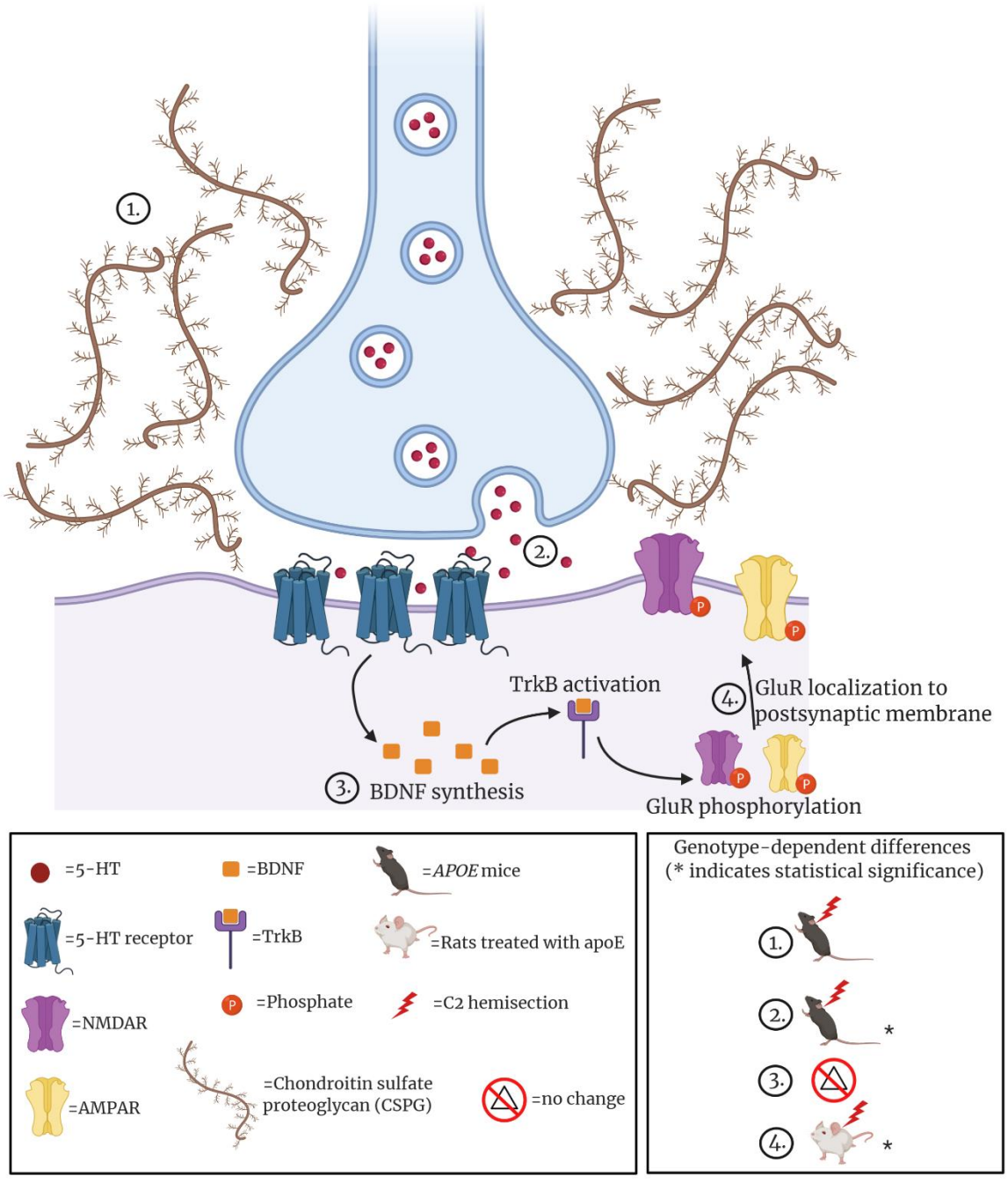

Figure 5.2 Graphic illustrating the signaling pathway that leads to manifestation of LTF.

Numbers indicate the molecules that were quantified in *APOE* targeted replacement mice or apoE-treated rats. The panel on the bottom right summarizes whether there were apoE isoform-dependent differences in the levels of these molecules: 1-2) Injured *APOE* mice displayed genotype-dependent differences in the amount of 5-HT density of the PNN around phrenic motor neurons. 3) ApoE isoforms did not influence the levels of BDNF in

apoE-treated rats or *APOE* mice. 4) ApoE4 was associated with a decline in synaptic NMDA receptors in C2 hemisected rats. (Created with BioRender.com)

APPENDICES

Appendix 1. List of Abbreviations:

ABCA1 = ATP binding cassette A1
AD = Autonomic dysreflexia
AIS = American Spinal Injury Association Impairment Scale
AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA = Analysis of variance
ApoE = Apolipoprotein E
ATP = Adenosine triphosphate
BDNF = Brain derived neurotrophic factor
CNS = Central nervous system
CPP = Crossed phrenic pathway
CSPG = Chondroitin sulfate proteoglycan
DP = Diaphragm pacing
ELISA = Enzyme-linked immunosorbent assay
GDNF = Glial cell-derived neurotrophic factor
HIV = Human immunodeficiency virus
HVR = Hypoxic ventilatory response
LDLR = Low density lipoprotein receptor
LTF = Long term facilitation
LTP = Long term potentiation
MP = Methylprednisolone
MRI = Magnetic resonance imaging
NADPH = Nicotinamide adenine dinucleotide phosphate
NIH = National Institutes of Health
NLI = Neurological level of injury
NMDA = N-methyl-D-aspartic acid
NMDA = NMDA receptor
NSAID = Nonsteroidal anti-inflammatory drug
NTS = Nucleus tractus solitarius
OCT = Optimal cutting temperature
PBS = Phosphate buffered saline
PFA = Paraformaldehyde
PMN = Phrenic motor neuron
PNN = Perineuronal net
RMANOVA = Repeated measures ANOVA
ROI = Region of interest
SCI = Spinal cord injury
TBI = Traumatic brain injury
TMS = Transcranial magnetic stimulation
VLDL = Very low density lipoprotein

Appendix 2. Copyright for published data

Data from Chapter 3 has been published in *eNeuro.* The following is their statement regarding use and reproduction of published material:

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VITA

Education

Doctor of Philosophy, Neuroscience **2016-Present**
University of Kentucky, Lexington, KY
Dissertation Advisor: Warren J. Alilain, PhD

Graduate Certificate in Anatomical Sciences Instruction **2018-2020**
University of Kentucky
Teaching Certificate in Gross Anatomy
Teaching Certificate in Neuroanatomy

Bachelor of Science, Biology **2012-2016**
Marshall University, Huntington, WV
Research Advisor: Elmer Price, PhD

Research Experience

Graduate Research Assistant, University of Kentucky **2016-Present**
Warren Alilain, PhD
Investigating the impact of apolipoprotein E genotype on the modulation of respiratory motor plasticity following cervical spinal cord injury

Undergraduate Research, Marshall University **2013-2016**
Elmer Price, PhD
Developing fibrin-based microenvironments for recruitment of neural stem cells

Positions in Professional Organizations

Diversity, Equity, and Inclusion Council, Dept. of Neuroscience 2020-Present
American Spinal Injury Association 2019-Present
Early Career Committee member
SCI Journal Club organizer, SCoBIRC 2018
Alpha Chi Sigma fraternity for professionals in chemistry 2014-2016
Treasurer of Gamma Eta chapter

Funding and Awards

$138,000 National Science Foundation Graduate Research Fellowship 2018-2021
$3,400 Graduate Student Incentive Program awarded through the University of Kentucky for receiving a nationally competitive award 2018-2020
$500 Therapeutic Intermittent Hypoxia Retreat Travel Award 2018
$500 International Symposium on Neural Regeneration Travel Award 2017

$12,500	University of Kentucky College of Medicine Fellowship for Excellence in Graduate Research	2017
$4,500	West Virginia NASA Space Grant Consortium Undergraduate Research Fellowship	2015
$8,000	Arch Coal Scholarship	2012-2016
$19,000	West Virginia Promise Scholarship	2012-2016
$5,100	Marshall University John Marshall Scholarship	2012-2016
$2,000	Governor's Honors Academy Scholarship	2012-2016
$5,000	Marshall University Presidential Scholarship	2012-2016

Conference Presentations

2019 Neuroscience Conference, Chicago, IL "Apolipoprotein E modulates respiratory motor plasticity following cervical spinal cord injury" Poster Presenter

2019 Experimental Biology Conference, Orlando, FL "Apolipoprotein E modulates respiratory motor plasticity following cervical spinal cord injury" Poster Presenter

2019 SCI 2020: National Institutes of Health Launching a Decade for Disruption in Spinal Cord Injury Research, Bethesda, MD "Apolipoprotein E modulates respiratory motor plasticity following cervical spinal cord injury" Poster Presenter

2018 Society for Neuroscience Conference, San Diego, CA "Apolipoprotein E as a barrier to respiratory motor plasticity" Poster Presenter

2018 Kentucky Spinal Cord and Head Injury Research Trust, Lexington, KY "Apolipoprotein E4 as a barrier to respiratory motor plasticity" Poster Presenter

2018 Therapeutic Intermittent Hypoxia Retreat, Gainesville, FL "The ApoE Gene as a Barrier to Respiratory Motor Plasticity" Platform presentation, Data Blitz Presenter

2017 International Symposium on Neural Regeneration, Asilomar, CA "Toward Precision Medicine After SCI: The Genetic Influence of ApoE on Respiratory Motor Plasticity" Platform presentation, Data Blitz Presenter

2017 Kentucky Neuroscience Institute Clinical Translational Research Symposium, Lexington, KY "Toward Precision Medicine After SCI: The Genetic Influence of ApoE on Respiratory Motor Plasticity" Platform Presentation

2017 International Spinal Research Trust meeting, London, UK: "Toward Precision Medicine After SCI: The Genetic Influence of ApoE on Respiratory Motor Plasticity" Poster Presenter

2015 Neuroscience Conference, Chicago, IL: "Fibrin-based microenvironments for in vitro studies of adult neurogenesis" Poster Presenter

2015 West Virginia IDeA Network of Biomedical Research Excellence, Summer Research Symposium, Huntington, WV, "Engineered Fibrin Implants that Recruit Endogenous Neural Stem Cells as Therapies for Brain Disorders". Poster Presenter

Publications

ARTICLES

Lydia E. Strattan, Daimen R. Britsch, Chris M. Calulot, Rachel S.J. Maggard, Erin L. Abner, Lance A. Johnson, Warren J. Alilain "Novel influences of sex and *APOE* genotype on spinal plasticity and recovery of function after spinal cord injury" *eNeuro.* 2021 (in press)

Cregg, J., Chu, K., **Hager, L.E.** Maggard, R.S.J., Stoltz, D.R., Eastman, B., Alilain, W.J., Philippidou, P., Landmesser, L.T., Silver, J. "A Latent Propriospinal Network Can Restore Diaphragm Function After High Cervical Spinal Cord Injury" *Cell Reports.* 2017

Carter, A.B., **Hager, L.E.**, Clark, A.R. and Price, E.M. "In Vivo Neural Tissue Engineering: Cylindrical Biocompatible Hydrogels That Create New Neural Tracts in the Adult Mammalian Brain." *Stem Cells and Development.* 2016

ONLINE CONTENT

Lydia E. Strattan, "Enhancing Breathing After Spinal Cord Injury Through Axonal Regeneration in Rats." *eNeuro.* 2020

ABSTRACTS

Lydia E. Strattan, Kyle J. Ritter, Chris M. Calulot, Daimen R. Britsch, Aaron L. Silverstein, Laura E. Mendenhall, Emily E. Huffman, Lance A. Johnson, Warren J. Alilain "Apolipoprotein E modulates respiratory motor plasticity following cervical spinal cord injury". Society for Neuroscience, Chicago, IL 2019

Jessica S. Newton, Emily H. Huffman, Daimen R.S. Britsch, **Lydia E. Strattan,** Rachel S.J. Maggard, Laura E. Mendenhall, Chris M. Calulot, Warren J. Alilain "Longitudinal analysis of gut microbiome composition after experimental cervical spinal cord injury". Society for Neuroscience, Chicago, IL 2019

Rachel S.J. Maggard, Christopher M. Calulot, Emily E. Huffman, Daimen R. Stoltz, **Lydia E. Hager**, Kyle J. Ritter, Brittany N. Turba, Jared D. Hoffman, Ai-Ling Lin, Lance A. Johnson, Warren J. Alilain "Human Apolipoprotein E Isoforms Influence Neurite Outgrowth and Regeneration In Vitro and Serotonergic Sprouting In Vivo". Society for Neuroscience, Chicago, IL 2019

Emily E. Huffman, Jessica S. Newton, Daimen R. Stoltz, **Lydia E. Hager**, Warren J. Alilain "Investigating the link between cervical spinal cord injury and acute lung injury" Society for Neuroscience, Chicago, IL 2019

Lydia E. Hager, Rachel S.J. Maggard, Daimen R. Stoltz, Kyle J. Ritter, Emily E. Huffman, Warren J. Alilain "Apolipoprotein E modulates respiratory motor plasticity following cervical spinal cord injury". Experimental Biology, Orlando, FL 2019

Lydia E. Hager, Rachel S.J. Maggard, Daimen R. Stoltz, Kyle J. Ritter, Emily E. Huffman, Brittany N. Turba, Warren J. Alilain "Apolipoprotein E as a barrier to respiratory motor plasticity". Society for Neuroscience, San Diego, CA, 2018.

Rachel S.J. Maggard, Christopher M. Calulot, **Lydia E. Hager**, Kyle J. Ritter, Brittany N. Turba, Jared D. Hoffman, Ai-Ling Lin, Lance A. Johnson, Warren J. Alilain "Human Apolipoprotein E Isoforms Differentially Influence Neurite Outgrowth and Regeneration". Society for Neuroscience, San Diego, CA, 2018

Lydia E. Hager, Rachel S.J. Maggard, Daimen R. Stoltz, Kyle J. Ritter, Brittany N. Turba, Warren J. Alilain "Toward Precision Medicine After SCI: The Genetic Influence of ApoE on Respiratory Motor Plasticity" International Spinal Research Trust, London, UK, 2017.

Warren J. Alilain, Daimen R. Stoltz, Kyle J. Ritter, **Lydia E. Hager**, Adam O. Ghoweri, Thomas E. Dick, Anthony F. DiMarco, Krzysztof E. Kowalski "Promoting Respiratory Motor Plasticity After Spinal Cord Injury Through High Frequency Stimulation (HF-SCS)" Abstract 6378, Experimental Biology, Chicago, IL, 2017.

Lydia E. Hager, Amanda R. Clark and Elmer M. Price, "Fibrin-based microenvironments for in vitro studies of adult neurogenesis" Abstract: 91.17/AA44, Society for Neuroscience, Washington, D.C., 2015.

Amanda R. Clark, **Lydia E. Hager** and Elmer M. Price, "Implantable fibrin cylinders that recruit new cells into the striatum and cortex" Abstract: 45.11/D25, Society for Neuroscience, Washington, D.C., 2015.

Amanda R. Clark, **Lydia E. Hager** and Elmer M. Price "Engineered Fibrin Implants that Recruit Endogenous Neural Stem Cells as Therapies for Brain Disorders" West Virginia IDeA Network of Biomedical Research Excellence, Summer Research Symposium