Respiratory pathology in the Optn⁻/⁻ mouse model of Amyotrophic Lateral Sclerosis

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A B S T R A C T

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder that results in death due to respiratory failure. Many genetic defects are associated with ALS; one such defect is a mutation in the gene encoding optineurin (OPTN). Using an optineurin null mouse (Optn⁻/⁻), we sought to characterize the impact of optineurin deficiency on respiratory neurodegeneration. Respiratory function was assessed at 6 and 12 mo of age using whole body plethysmography at baseline during normoxia (FiO₂: 0.21; N₂ balance) and during a respiratory challenge with hypoxia and hypercapnia (FiCO₂: 0.07, FiO₂: 0.10; N₂ balance). Histological analyses to assess motor neuron viability and respiratory nerve integrity were performed in the medulla, cervical spinal cord, hypoglossal nerve, and phrenic nerve. Minute ventilation, peak inspiratory flow, and peak expiratory flow are significantly reduced during a respiratory challenge in 6 mo Optn⁻/⁻ mice. By 12 mo, tidal volume is also significantly reduced in Optn⁻/⁻ mice. Furthermore, 12mo Optn⁻/⁻ mice exhibit hypoglossal motor neuron loss, phrenic and hypoglossal dysmyelination, and accumulated mitochondria in the hypoglossal nerve axons. Overall, these data indicate that Optn⁻/⁻ mice display neurodegenerative respiratory dysfunction and are a useful model to study the impact of novel therapies on respiratory function for optineurin-deficient ALS patients.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that results in paralysis and death from respiratory failure. As ALS progresses, diaphragm and intercostal muscle weakness leads to inadequate ventilation and respiratory insufficiency. In addition to weakness of these respiratory muscles, the genioglossus muscle of the tongue progressively deteriorates. This bulbar muscle regulates tongue shape, stiffness, and position and is crucial for upper airway patency. Thus, tongue muscle weakness results in dysarthria and dysphagia leading to recurrent aspiration, choking, and aggravation of respiratory disease. The progressive bulbar and diaphragm weakness is a consequence of hypoglossal and phrenic motor unit degeneration. As the disease progresses, motor neuron axons retract from tongue, diaphragm, and intercostal muscle fibers, which result in respiratory muscle weakness and atrophy.

Throughout the last 25 years, genome analysis in ALS patients identified mutations in more than 40 genes. In 2010, mutations in the gene OPTN, which encodes the protein optineurin (OPTN), were first identified in six Japanese ALS patients. OPTN is a multifunctional adaptor protein involved in several, overlapping cellular pathways - mitophagy, NFκB-regulated inflammation, and necroptosis. By promoting mitophagy and inhibiting chronic inflammation, OPTN is a key protein in cell homeostasis. Cellular homeostasis is particularly important for non-dividing cells such as motor neurons. The loss of OPTN also promotes necroptosis through its interaction with Receptor Interacting Serine/Threonine Kinase 1 (RIPK1) resulting in dysmyelination and axonal degeneration. Patients with OPTN mutations have a typical age of onset (30-60 years) for ALS, but most display a slower disease progression with delayed respiratory failure. Since 2010, OPTN mutations have been identified in 3-4% of Japanese fALS patients and up to 14% of Moroccan patents. Mutations that result in loss of the functional OPTN protein lead to disrupted autophagy, necroptosis, and inflammation which result in harmful effects that leads to ALS pathogenesis. Interestingly, individuals with loss of function mutations in OPTN develop tongue weakness early in the disease course that leads to choking, failure to control secretions, upper airway obstruction, and aspiration pneumonia. As the disease progresses,
phrenic nerve and motoneurons are progressively affected and death is ultimately due to respiratory failure. \cite{5,16-19}

While much is known about how OPTN behaves molecularly, its role in ALS-associated respiratory dysfunction is unknown. The goal of this study was to characterize respiratory deficiency in a novel Optn	extsuperscript{-/-} mouse as a model of ALS. \cite{20}. The overall hypothesis is that OPTN-deficiency induces neurodegeneration resulting in respiratory neuropathology and insufficiency. Overall, our findings indicate that the Optn	extsuperscript{-/-} mouse is an ideal animal model for studying mechanisms of respiratory failure in patients with optineurin-deficient ALS.

2. METHODS

2.1. Mice

All mice were approved by the Duke University Institutional Animal Care and Use Committee (IACUC). C57BL6/J, wildtype (WT), mice were obtained from the Jackson Laboratory. Optn	extsuperscript{-/-} mice were generated by Dr. Tseng at Duke University \textsuperscript{1}. All mice were housed at the Duke University Division of Laboratory Animal Resources.

2.2. Respiratory Analysis

Whole body plethysmography (WBP) was performed as previously described \cite{22} at 6 and 12 mo in the WT and Optn	extsuperscript{-/-} mice (N = 8-11 mice per group). Briefly, unanesthetized, unrestrained mice were placed in a Plexiglas chamber (DSI, St. Paul, MN); data was collected and analyzed using FinePointe Software. Ventilation was monitored under normoxia (FiO\textsubscript{2}: 0.21; N\textsubscript{2} balance) for 1.5 hours, within which a five-minute period of regular breathing was selected as the baseline. Following the period of normoxia, mice were exposed to a hypercapnic and hypoxic (FiO\textsubscript{2}: 0.07, FiO\textsubscript{2}: 0.10; N\textsubscript{2} balance) respiratory challenge for 10 minutes. Mice were then returned to normoxic air. Apneic events were also measured during the baseline and represent averages of 15 second intervals. For breaths to be defined as an apnea they needed to meet one of the following two criteria: (1) be 1.5 seconds in duration or longer, AND/OR (2) be 200% longer than the previous breath.

2.3. Motor Neuron Immunohistochemistry

Hypoglossal and phrenic motor neurons were labeled with the retrograde tracer, cholera toxin subunit β (CT-β), as previously described \cite{23,24}. A 0.2% solution of CT-β (Millipore-Sigma, C9903) in Lactated Ringers was delivered through intralungal and intrapleural injections to 12 mo old mice (N = 2 – 3 per tissue per genotype). 72 hours after injection, the brainstem and spinal cords on bloc within the bone were harvested and fixed in 4% paraformaldehyde (PFA) for 24 hours. The brainstems and spinal cords were extracted from the soft tissue and bone, then fixed in 4% PFA for an additional 24 hours. Extracted brainstems and spinal cords were then cryopreserved in 30% sucrose then embedded in OCT. The medulla and cervical spinal cord were sectioned at 40 μm. Every second section from the hypoglossal region of the medulla and from C3-C5 within the cervical spinal cord was stained with an anti-cholera toxin antibody (List-Biologicals, 703). A biotinylated secondary antibody (VECTASTAIN ABC kit, Vector Laboratories, 1:200) exposed with 3,3’-diaminobenzidine (DAB) was applied. Sections were counterstained with cresyl violet, then imaged with a Leica DMRA2 Compound Microscope with Open Lab Software at 10x magnification. Motor neurons in the XII and phrenic motor pools were independently counted by two lab members who were blinded to the genotype of the mice.

2.4. Nerve g-Ratio Quantification

Phrenic and hypoglossal nerves of 12 mo old mice (N = 3-4 mice per group) were harvested and placed in 2.5% glutaraldehyde and 0.10 M sodium cacodylate. Samples were subsequently washed in 1.0% osmium tetroxide and then placed into en bloc stain (1% uranyl acetate). Next, they were dehydrated in acetone and epoxy resin (EPON). Finally, the samples were embedded in Beem capsules and semi-thin sectioned (1 μm), and stained with 1% toluidine blue-1% sodium borate solution by the Duke University Electron Microscopy Core. Slides were imaged on a Leica DMRA2 Compound Microscope with Open Lab Software at 20x magnification. G-ratios of the cross-sections were quantified using a public downloadable ImageJ plugin \textsuperscript{25}. The g-ratio was determined to be the ratio of the inner axonal diameter to the total outer axonal diameter with surrounding myelin sheath of each axon fiber. Each area was manually outlined on 100 randomly selected axons for the hypoglossal nerve, and 50 randomly selected axons for the phrenic nerve.

2.5. Mitochondrial Analysis

Processed and blocked phrenic and hypoglossal nerves from above (2.4) were ultrathin sectioned (~60 nm) on a Reichert Ultracut E ultramicrotome, and post-stained in 2% uranyl acetate and SATO’s lead stain. Images were captured using a Philips CM12 transmission electron microscope. Ultrastructural analysis was conducted using Imagej 2.0 to quantify mitochondria per axon, axonal area, and mitochondrial area. Mitochondria were required to have a minimum diameter of 0.16 μm to be counted \textsuperscript{26}.

2.6. Statistical Analysis

All data were analyzed in GraphPad Prism Software. The WBP breathing parameters were analyzed using a two way ANOVA with repeated measures and post-hoc analysis was performed using a Bonferroni correction. Apnea, body weight, G-ratio, and mitochondrial size quantification, were analyzed using a Student’s t-test. Data are reported as mean ± SEM. For all statistical analyses, significance is defined as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. RESULTS

3.1. Respiratory deficiency in Optn	extsuperscript{-/-} mice is present by 6 months of age

Awake, spontaneous ventilation was assessed at baseline during normoxic breathing (FiO\textsubscript{2}: 0.21; nitrogen balance) and during a respiratory challenge with hypoxia and hypercapnia (FiO\textsubscript{2}: 0.10; FICO\textsubscript{2}: 0.07; nitrogen balance). At baseline, there are no differences in frequency (f), tidal volume (TV), minute ventilation (MV), peak inspiratory flow (PIF) or peak expiratory flow (PEF) between WT and Optn	extsuperscript{-/-} mice at 6 or 12 mo of age (p > 0.05). However, during the respiratory challenge, 6 mo old Optn	extsuperscript{-/-} mice have decreased MV (p = 0.0004), PIF (p = 0.0007), and PEF (p = 0.0003) (Fig. 1) compared to WT mice. Furthermore, frequency is decreased (p = 0.05) but TV is not significantly decreased (p = 0.07). At 12 mo of age, Optn	extsuperscript{-/-} mice develop a progressive deterioration in TV (p = 0.02), and maintain a significant decrease in MV (p = 0.003), PIF (p = 0.01), and PEF (p < 0.0001) (Fig. 2). Frequency is not different between WT and Optn	extsuperscript{-/-} mice at 12 mo of age (p = 0.21). We also noted that growth of Optn	extsuperscript{-/-} mice is reduced compared to WT controls by one year of age (Supplemental Fig. 1).

Since ALS patients also have significant respiratory apnea, we assessed the apneic events in Optn	extsuperscript{-/-} mice and compared this to WT mice. Both WT and Optn	extsuperscript{-/-} mice had several apneic events which has been documented in C57BL6 mice. \cite{27}. However, compared to WT controls, 6-mo-old Optn	extsuperscript{-/-} mice trend toward longer apneic events, which is lost in 12-mo-old mice (Fig. 3A and B) (6-mo: p = 0.053; 12-mo: p = 0.29). In WT controls, apneas last the duration of 2 – 3 breaths, whereas an apnea in Optn	extsuperscript{-/-} mice can last up to 3 – 5 breaths, as observed in the representative tracing (Fig. 3C).
3.2. Fewer motor neurons are present in respiratory centers in Optn−/− mouse brainstem

Mice were euthanized at 12 mo of age for analysis of respiratory motor neuron and nerve axon health. Compared to WT mice, Optn−/− mice have fewer motor neurons in the hypoglossal motor nucleus, which controls the genioglossus muscle, at 12 mo of age (Fig. 4A and C). We also quantified motor neurons in the putative phrenic motor nucleus in the ventral horn of the cervical spinal cord which control the diaphragm (Fig. 3B). Unlike changes in the hypoglossal motor nucleus, phrenic motor neuron survival was unaffected (p = 0.45).

3.3. Optn−/− mice have disorganized myelin sheaths

The g-ratio, a measure of myelination in relation to axon size, is smaller in both the hypoglossal and phrenic nerves from Optn−/− mice (hypoglossal: p = 0.0245; phrenic: p = 0.0001), compared to WT mice. This decreased g-ratio indicates thicker myelin sheaths (Fig. 5). Qualitative analysis of electron micrographs indicate that the smaller g-ratio is due to disorganized and frayed myelin, rather than an increase in myelin (Fig. 6C and F). In comparison, WT mice exhibit thinner and more compact myelin sheaths and thus larger g-ratios.

3.4. Accumulation of mitochondria in Optn−/− axon at 12 mo

Mitochondrial health within hypoglossal and phrenic motor neuron axons was assessed using transmission electron microscopy. In 12-mo-old Optn−/− hypoglossal motor neuron axons, there are more mitochondria (p < 0.0001), which qualitatively appear swollen, although not significantly (p = 0.053) (Fig. 6A–C). In contrast, mitochondria in axons from WT mice demonstrate normal size and appearance. Interestingly, in the phrenic axons neither mitochondria accumulation (p = 0.21) nor mitochondrial enlargement (p = 0.75) in Optn−/− mice is observed (Fig. 6D–F).

4. DISCUSSION

The primary findings of this study are that Optn−/− mouse exhibit respiratory insufficiency, hypoglossal motor neuron loss, and hypoglossal nerve abnormalities. Respiratory deficits in awake, unrestrained, spontaneously breathing mice are evident by 6 mo of age and continue through 12 mo of age. Interestingly, in the Optn−/− mouse there are fewer compact myelin sheaths and thus larger g-ratios.
motor neurons in the hypoglossal motor nucleus but not in the phrenic motor nucleus. Furthermore, there is myelin disorganization along the hypoglossal and phrenic nerves, and accumulation of enlarged mitochondria in hypoglossal nerve axons. Our findings suggest that loss of optineurin leads to respiratory insufficiency in the Optn\textsuperscript{−/−} mouse and that this pathology is more apparent in the hypoglossal motor pool.

4.1. ALS & Respiratory Dysfunction

Despite overwhelming evidence of respiratory distress in ALS patients, only a few studies report respiratory deficits in the most common mouse model, the SOD1\textsuperscript{G93A} mouse\textsuperscript{28,29}. In the SOD1\textsuperscript{G93A} mouse, TV, MV, PIF, and PEF are maintained through 18 weeks of age, but drop off substantially compared to nontransgenic littermate controls at endstage (~20 weeks of age). Similar to our findings in the Optn\textsuperscript{−/−} mouse, these differences are observed only when SOD\textsuperscript{G93A} mice are challenged, and not at baseline\textsuperscript{28}. However, in contrast to the SOD1\textsuperscript{G93A} mice, disease onset is much later and progression is much slower in the Optn\textsuperscript{−/−} mice. Our data show that respiratory deficits are apparent by 6 mo of age. When exposed to a respiratory challenge, MV, PIF, and PEF are all reduced in the SOD\textsuperscript{G93A} mice compared to the WT controls. A decrease in PIF indicates weakness of the inspiratory muscles – namely the diaphragm – whereas a decrease in PEF is a reflection of weakened expiratory muscles such as the internal intercostal and accessory respiratory muscles\textsuperscript{30}. At 12 mo of age, significant differences between WT & Optn\textsuperscript{−/−} mice are maintained for MV, PIF, and PEF, but TV deteriorates with age. This slow progression is also observed in other Optn\textsuperscript{−/−} mouse models\textsuperscript{13}. Another potential reason for this unexpected finding is that in ALS due to OPTN deficiency, patients show no symptoms initially and many then develop a slowly progressive disease until end stage when severe respiratory pathology occurs. At 12mo, the Optn\textsuperscript{−/−} mice have not yet reached end stage and respiratory pathology may require additional environmental stressors to instigate disease progression. These stressors may include hypoxia, viral infections, or occupational stressors that can...
exacerbate and trigger further respiratory degeneration\textsuperscript{31}. Further studies will need to investigate the impact of both environmental stressors and age on disease onset and progression.

To investigate the cause of the observed respiratory deficits, we examined respiratory motor neuron loss in the medulla and cervical spinal cord for loss of motor neurons which is a major hallmark of ALS. Specifically, we focused on hypoglossal motor neurons in the medulla, which are responsible for maintaining airway patency, and phrenic motor neurons in the cervical spinal cord, which innervate the diaphragm. These neurons and nerves are crucial for proper ventilation\textsuperscript{2}. Loss of both hypoglossal and phrenic motor neurons is observed in the SOD1\textsuperscript{G93A} mouse at endstage\textsuperscript{32,33}. Here, we only saw loss of motor neurons in the hypoglossal motor nucleus and not in the phrenic motor pool. This preservation of phrenic motor neurons at 12 mo may explain the lack of severe respiratory decline from 6 to 12 mo. We hypothesize that this lack of phrenic motor neuron pathology at 12 mo occurs because Optn\textsuperscript{−/−} mice are not yet at end stage. Thus, similar to patients with OPTN deficiency, the bulbar muscles are affected early, and phrenic motor neuron preservation delays respiratory failure for several years after diagnosis\textsuperscript{34,35}. However, at 12 mo the phrenic nerve exhibits significant abnormalities in myelination which can explain the deteriorating tidal volume. Similar to our findings, Ito et al. did not find reduced numbers of motor neurons in the spinal cord of Optn\textsuperscript{−/−} mice; however, they only examined the lumbar motor neurons, and did not examine motor neurons rostral of the lumbar region.

Despite the preserved phrenic motor neuron counts, there was evidence of abnormal myelination in both the hypoglossal and phrenic nerve. This is congruent with the findings of Ito et al. who reported decompaction of the myelin sheath and normal myelination in the spinal cords of Optn\textsuperscript{−/−} mice\textsuperscript{13}. Together, these indicate that myelin disorganization occurs in both the phrenic and hypoglossal nerves, whereas the loss of motor neurons is more prominent in the hypoglossal motor pool. It is unclear why the hypoglossal motor neurons are more vulnerable early in disease, but these findings are consistent with the clinical phenotype\textsuperscript{34–36}. Future studies that investigate instigating stressors might unmask phrenic motor neuron loss and further pathology.

ALS patients experience an increase in central sleep apnea. We
examined the presence of apneas in these mice, and both the WT and the Optn<sup>−/−</sup> mice had increased apnea. Although there was a trend towards increased time in apnea in Optn<sup>−/−</sup> mice, we did not find significant differences between WT and the Optn<sup>−/−</sup> mice. This lack of significant difference in apnea is mostly likely secondary to the increased prevalence of apneas in C57BL/6J mice<sup>65</sup>.

4.2. Optineurin, Mitophagy, and ALS

Dysfunction of the autophagy pathway is a major cause of molecular ALS pathology<sup>33</sup>. OPTN plays several roles in autophagy as a scaffold for autophagosome elongation and maturation machinery, and as a cargo adaptor protein<sup>29</sup>–<sup>30</sup>. Reduced autophagy of mitochondria (mitophagy) results in accumulation of non-functional mitochondria and reactive oxidative species<sup>20</sup>–<sup>21</sup>. Interestingly, we discovered that Optn<sup>−/−</sup> hypoglossal motor neuron axons have an accumulation of mitochondria. The trend towards increased mitochondrial size indicates swelling, which is a hallmark of dysfunction in these organelles<sup>62</sup> – suggesting potential axonal transport deficiency or, more likely, impaired mitophagy<sup>64</sup>. Accordingly, mitochondrial abnormalities have been well-documented in the SOD1<sup>G93A</sup> mouse model of ALS as well<sup>15</sup>. Similar to the Optn<sup>−/−</sup> mice, motor axons in SOD1<sup>G93A</sup> contain swollen mitochondria that have been shown to contribute to the onset of motor neuron degeneration<sup>40</sup>.

4.3. Conclusion

Overall, we demonstrate that this novel Optn<sup>−/−</sup> mouse displays respiratory dysfunction which is similar to that observed in ALS patients with OPTN deficiency. This respiratory pathophysiology includes respiratory insufficiency during a hypoxia and hypercapnic challenge, hypoglossal and phrenic nerve myelin disorganization with loss of hypoglossal motor neuron cell bodies, and an accumulation of abnormal mitochondria in the hypoglossal nerve axons. Given the slow progression and mild ALS phenotype, Optn<sup>−/−</sup> mice provide an important opportunity and an ideal tool to evaluate novel therapeutics as well as identify stress-dependent mechanisms that exacerbate neurodegeneration and respiratory function in ALS.

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CRediT authorship contribution statement

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Appendix A. Supplementary data

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