



# Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome

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The most common pediatric mitochondrial disease is Leigh syndrome, an episodic, subacute neurodegeneration that can lead to death within the first few years of life, for which there are no proven general therapies. Mice lacking the complex I subunit, *Ndufs4*, develop a fatal progressive encephalopathy resembling Leigh syndrome and die at  $\approx 60$  d of age. We previously reported that continuously breathing normobaric 11% O<sub>2</sub> from an early age prevents neurological disease and dramatically improves survival in these mice. Here, we report three advances. First, we report updated survival curves and organ pathology in *Ndufs4* KO mice exposed to hypoxia or hyperoxia. Whereas normoxia-treated KO mice die from neurodegeneration at about 60 d, hypoxia-treated mice eventually die at about 270 d, likely from cardiac disease, and hyperoxia-treated mice die within days from acute pulmonary edema. Second, we report that more conservative hypoxia regimens, such as continuous normobaric 17% O<sub>2</sub> or intermittent hypoxia, are ineffective in preventing neuropathology. Finally, we show that breathing normobaric 11% O<sub>2</sub> in mice with late-stage encephalopathy reverses their established neurological disease, evidenced by improved behavior, circulating disease biomarkers, and survival rates. Importantly, the pathognomonic MRI brain lesions and neurohistopathologic findings are reversed after 4 wk of hypoxia. Upon return to normoxia, *Ndufs4* KO mice die within days. Future work is required to determine if hypoxia can be used to prevent and reverse neurodegeneration in other animal models, and to determine if it can be provided in a safe and practical manner to allow in-hospital human therapeutic trials.

hypoxia | mitochondria | Leigh syndrome | neurodegeneration | oxygen

Defects in mitochondrial function, owing to mutations either in the nuclear genome or in the mitochondrial DNA, result in severe diseases that can present at any point from infancy through adulthood (1). The most common biochemical class of mitochondrial disorders arises from genetic mutations affecting the mitochondrial respiratory chain, with an incidence of  $\sim 1$  in 4,300 live births (2). The management of these disorders is challenging in part because of their clinical and genetic heterogeneity. Virtually any organ system can be impacted, and more than 250 genes encoding mitochondrial proteins are known to be disease-causing (3). Of these, at least 150 can underlie disorders of oxidative phosphorylation. The mainstay of therapy involves using vitamin mixtures with little or no proven efficacy, as well as avoiding drugs known to be mitochondrial toxins.

Leigh syndrome, the most common pediatric manifestation of mitochondrial disease, is characterized by bilaterally symmetric lesions in the gray matter of the brainstem, basal ganglia, or cerebellum. It affects  $\sim 1$  in 40,000 live births and can be due to mutations in any of 75 different genes (4). Children with this disorder often become hypotonic and may develop vision and hearing loss. Although the course of disease can vary widely among individuals, severely affected children can experience developmental delay and ultimately succumb to respiratory failure

within the first few years of life. These patients are typically healthy at birth but then experience a neurometabolic crisis, often in the context of a febrile illness, resulting in psychomotor regression and death within the first few years of life. Diagnosis is based on clinical presentation and classic findings on T2-weighted brain magnetic resonance imaging (MRI). A small subset of these disorders are related to defects in vitamin transport and alleviated with dietary supplementation, such as in the case of riboflavin, thiamine, and biotin deficiencies (5, 6); however, there are no proven therapies for the vast majority of these disorders. Several new experimental strategies are currently under investigation in preclinical and clinical settings, including agents that target oxidative stress, the mechanistic target of rapamycin pathway, NAD<sup>+</sup> pool sizes, and complex I bypass (idebenone) (7–10).

We recently demonstrated the therapeutic potential of hypoxia in preventing mitochondrial disease. We reported that chronically exposing the *Ndufs4* mouse model of Leigh syndrome (11, 12) to breathing 11% O<sub>2</sub> at normobaria before disease onset markedly extended the lifespan, improved behavior, and prevented neurodegeneration (13). Furthermore, we demonstrated that breathing 55% O<sub>2</sub>, a level that is well tolerated by WT mice, led to rapid death of the diseased mice.

## Significance

Inherited or acquired defects in mitochondria lead to devastating disorders for which we have no effective general therapies. We recently reported that breathing normobaric 11% O<sub>2</sub> prevents neurodegeneration in a mouse model of a pediatric mitochondrial disease, Leigh syndrome. Here we provide updated survival curves of mice treated with varying doses of oxygen and explore eventual causes of death. We explore alternative hypoxia regimens and report that neither intermittent nor moderate hypoxia regimens suffice to prevent neurological disease. Finally, we show that hypoxia can not only prevent, but also reverses the brain lesions in mice with advanced neuropathology. Our preclinical studies will help guide future clinical studies aimed at harnessing hypoxia as a safe and practical therapy.

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Conflict of interest statement: V.K.M., W.M.Z., and I.H.J. are listed as coinventors on a patent application submitted by Massachusetts General Hospital on the therapeutic uses of hypoxia for mitochondrial disease.

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The goal of our present study was to build on this initial work (13) to better understand the disease course and pathophysiology of these mice when breathing different O<sub>2</sub> levels, and to explore alternative hypoxia regimens. We provide an update of the survival curves under hypoxia and carefully assess how variations of O<sub>2</sub> concentration and exposure duration affect organ pathology. We show that intermittent or more moderate hypoxia regimens are ineffective, and report that the effects of chronic breathing of 11% O<sub>2</sub> are not durable if mice return to breathing 21% O<sub>2</sub>. Importantly, we demonstrate that breathing 11% O<sub>2</sub> can not only prevent, but also reverse, established neurodegenerative disease in this murine model.

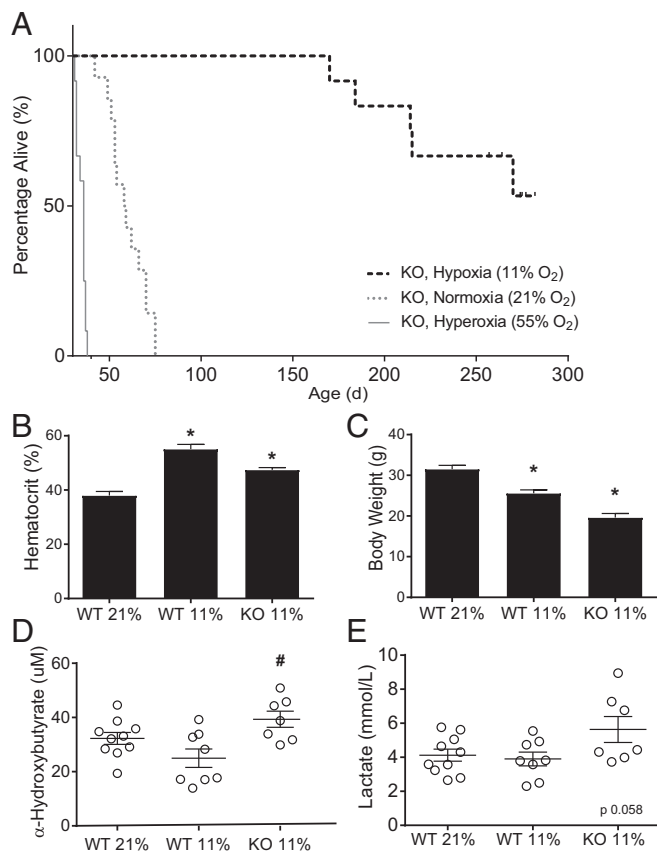
## Results

**Breathing 11% O<sub>2</sub> Prevents the Development of Brain Lesions Throughout the Life of Ndufs4 KO Mice.** To investigate the therapeutic potential of hypoxia treatment on mitochondrial dysfunction, we studied whether hypoxia is capable of preventing the neurodegenerative disease rather than simply delaying its onset (Fig. 1*A–E*). KO and WT controls were randomized to breathe either 21% or 11% O<sub>2</sub> starting at 30 d of age. As reported previously (13), our initial cohort of control KO mice breathing 21% O<sub>2</sub> all died between 42 and 75 d (median, 58 d). In contrast, KO mice breathing 11% O<sub>2</sub> were still alive at 170 d (13). Here we report an updated survival curve, showing that KO mice breathing 11% O<sub>2</sub> had an overall median survival duration of 270 d (log-rank,  $P < 0.0001$  vs. 21% O<sub>2</sub> controls) (Fig. 1*A*). To investigate the chronic physiological adaptation to hypoxia, we measured the venous hematocrit of mice after 9 mo of breathing 11% O<sub>2</sub>. Venous hematocrit was elevated, albeit to a lesser extent, in KO mice compared with WT controls ( $47 \pm 2\%$  vs.  $55 \pm 5\%$ , respectively;  $P < 0.05$ ;  $n = 8$ ) (Fig. 1*B*). The circulating plasma disease biomarkers lactate and  $\alpha$ -hydroxybutyrate are elevated in humans with Leigh syndrome and in the Ndufs4 mouse model (14). In our original report (13), both markers were partially normalized in KO mice breathing 11% oxygen. At 250 d,  $\alpha$ -hydroxybutyrate trended toward a partial rescue in KO mice breathing 11% oxygen ( $39 \pm 8 \mu\text{M}$ ;  $n = 7$ ) relative to normoxic-breathing KO mice at day 50 (13), although the difference was not statistically significant (Fig. 1*D*). At 250 d, plasma lactate levels in KO mice breathing 11% O<sub>2</sub> were similarly ameliorated (Fig. 1*E*) relative to normoxic-breathing KO mice at 50 d (13).

To study whether hypoxic breathing delays death by delaying the onset of CNS lesions, we performed T2-weighted brain MRI studies of lightly anesthetized mice chronically breathing either 21% or 11% O<sub>2</sub> ( $n = 4$ ). KO mice breathing 21% O<sub>2</sub> developed lesions in the posterolateral portion of the brainstem, localized to the vestibular nuclei. Lesions became evident at 55 d of age (Fig. 2*B*). In contrast, brain MRI scans performed in KO mice breathing 11% O<sub>2</sub> showed no signs of neurologic lesions even at 250 d.

We next assessed neuropathology in the same cohort of mice. Brains were stained for the inflammatory activation marker Iba-1 (Fig. 2*A*). KO mice breathing 21% O<sub>2</sub> displayed significant microglial activation in both the cerebellum and olfactory bulb (OB) by 50 d. In contrast, KO mice breathing 11% O<sub>2</sub> displayed no accumulation of Iba-1 in the cerebellum, OB, or brainstem at 250 d. In the latter group, brain sections were comparable to those of hypoxic, age-matched WT mice ( $n = 4$ ). These findings suggest that the cause of death in older, hypoxia-treated KO mice might differ from the progressive neurodegenerative disease of normoxia-treated KO mice. This led us to explore the functional alterations of other organs known to be involved in mitochondrial disease pathology.

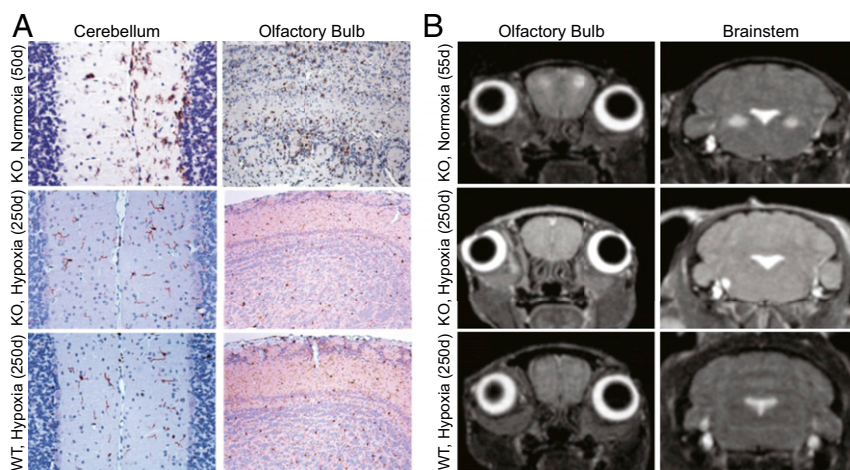
**Ndufs4 KO Mice Breathing 11% O<sub>2</sub> for >150 d Exhibit Mild Left Ventricular Dysfunction.** A common presentation of pediatric mitochondrial disease is cardiomyopathy, with left ventricular (LV) dysfunction negatively affecting prognosis in up to 20% of cases



**Fig. 1.** Survival, hematocrit, and disease markers in older Ndufs4 KO mice breathing 11% O<sub>2</sub>. (*A*) Survival rates of mice breathing various oxygen levels. Hypoxia-treated Ndufs4 KO mice were all alive at 170 d ( $n = 12$ ). The experiment was interrupted once 50% survival was achieved. All WT mice breathing 11% and 21% O<sub>2</sub> ( $n = 14$  per group) survived for 300 d (Kaplan–Meier log-rank between KO at 11% O<sub>2</sub> and KO at 21% O<sub>2</sub>,  $P < 0.0001$ , HR, 7.58; 95% CI, 2.75–20.9,  $n = 12$  per group). (*B*) After 30 wk of treatment, hematocrit was elevated in KO ( $n = 7$ ) and WT mice ( $n = 8$ ) breathing 11% O<sub>2</sub>. (*C*) Body weight of KO ( $n = 7$ ) and WT ( $n = 9$ ) controls at age 250 d. (*D* and *E*) Plasma levels of  $\alpha$ -hydroxybutyrate (*D*) and lactate (*E*) in hypoxic KO mice at age 250 d, compared with age-matched WT mice breathing 21% or 11% O<sub>2</sub>. Data are mean  $\pm$  SD. \* $P < 0.05$  vs. WT, 21% O<sub>2</sub>; # $P < 0.05$  vs. WT, 11% O<sub>2</sub>, one-way ANOVA with Bonferroni's correction.

(15). The pathogenesis of cardiac pathology is incompletely understood. The sudden death of some of our >170-d-old KO mice breathing 11% O<sub>2</sub> led us to investigate their cardiac function (Fig. 3*A–F*). To do so, we obtained echocardiograms in mildly sedated mice at 200 d (Fig. 3*A* and *C*). Using ultrasound, we detected decreased LV fractional shortening in KO mice compared with WT mice breathing 11% O<sub>2</sub> at 200 d ( $37 \pm 4\%$  vs.  $57 \pm 9\%$ ;  $P < 0.05$ ;  $n = 6$  in each group) (Fig. 3*A* and *C*).

We also examined ventricular function by MRI at 9.4 T with a gradient echo cine sequence (Fig. 3*F*) (16). The age-matched WT mice were anesthetized with 1–2% isoflurane, whereas the KO mice were anesthetized using 0.3% isoflurane owing to their increased sensitivity to anesthetic agents (17). Scans were performed while the mice were breathing 21% O<sub>2</sub>. Body temperature was maintained in the physiological range with a warm air blower. Heart rate averaged  $\sim 400$  bpm in WT mice and 300 bpm in KO mice. LV function assessed by cardiac MRI demonstrated a reduced LV ejection fraction in KO mice treated by breathing 11% O<sub>2</sub> at >200 d of age, compared with WT controls breathing 11% O<sub>2</sub> at the same age ( $56 \pm 7\%$  vs.  $69 \pm 2\%$ ;  $P < 0.05$ ;  $n = 3$  in each group) (Fig. 3*D*), confirming the echocardiographic findings. Assessment of right



**Fig. 2.** Absence of neurodegenerative pathology in 250-d-old hypoxia-treated *Ndufs4* KO mice. (A) Representative images with staining for the microglial activation marker Iba-1 ( $n = 3$ ). Normoxic KO mice at 50 d show a significant inflammatory response in the cerebellum and OB. Analogous images in 250-d-old hypoxic KO mice and WT mice do not show brain inflammation. (B) Axial head MRI images showing bilateral, symmetrical hyperintense lesions in the OB (Left) and brainstem (Right) of normoxic-breathing KO mice at 55 d. These lesions were not present in hypoxic KO mice at 250 d.

ventricular ejection fraction also revealed no significant differences between KO and WT mice ( $58 \pm 1\%$  vs.  $58 \pm 1\%$ ;  $P = 0.97$ ;  $n = 3$  in each group) (Fig. 3E). Of note, ECG sporadically revealed intermittent atrioventricular blockade in hypoxia-treated KO mice under isoflurane anesthesia. Furthermore, no differences in LV fractional shortening were detected between hypoxia-treated KO mice at 50 d and age-matched KO controls breathing 21%  $O_2$  (Fig. 3B).

**Breathing 55%  $O_2$  Causes Acute Lung Edema and Isolated OB Lesions in *Ndufs4* KO Mice.** To further understand the critical role of inhaled oxygen concentration on mitochondrial disease progression, we investigated the physiological effects of breathing 55%  $O_2$ . We previously reported the remarkable sensitivity of KO mice to mild hyperoxic breathing (13). On exposure to breathing 55%  $O_2$ , KO mice died between 48 h and 10 d, with a median survival of 5 d. To assess whether supplemental  $O_2$  exacerbates the original neurologic disease, we obtained brain MRI scans in KO mice that had been breathing 55%  $O_2$  for 24 h. Imaging showed hyperintense lesions in the OB, but no lesions in the brainstem (Fig. 4D), suggesting augmented  $O_2$  sensitivity of the olfactory cells of mice exposed to hyperoxia via the nasal epithelium, but not in the brainstem via the arterial circulation.

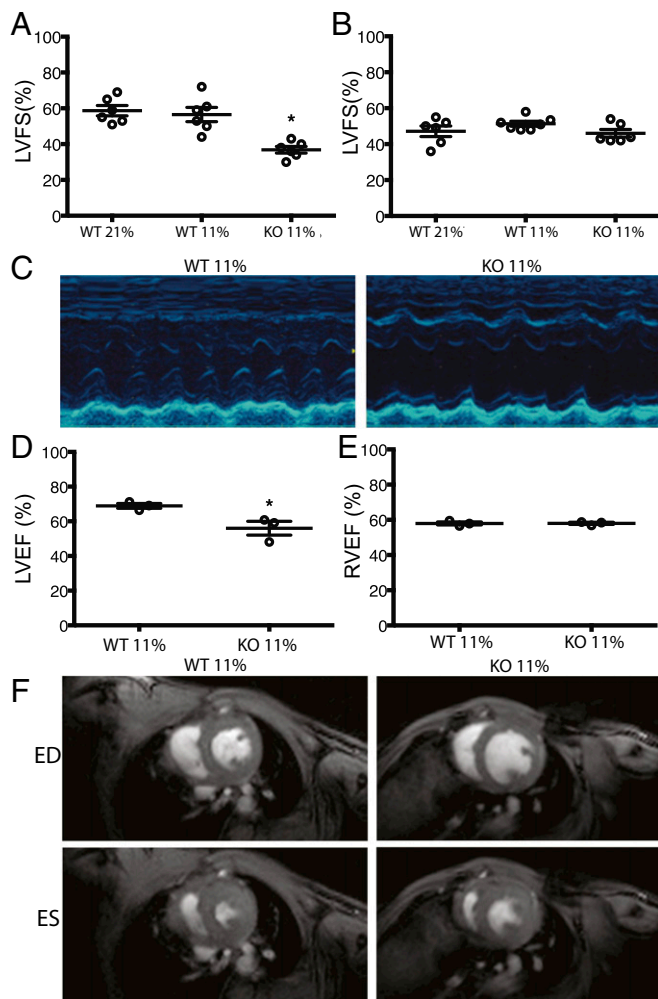
We then focused our attention on the lung, an organ known to be sensitive to supplemental  $O_2$  toxicity. It is known that breathing 100%  $O_2$  results in pulmonary edema within several days in WT mice (18). Thus, we wondered whether similar pathology would ensue at a more moderate hyperoxia exposure in KO mice. To estimate pulmonary edema, we assessed the wet-to-dry lung weight (WD) ratio (19). Mice were exposed to 55%  $O_2$  for 24 h starting at 30 d of age. The WD ratio was elevated, at  $5.63 \pm 0.6$  in KO mice and  $4.38 \pm 0.02$  in WT controls ( $P < 0.001$ ;  $n = 7$ ) (Fig. 4A), indicating significant acute pulmonary edema. Furthermore, lung myeloperoxidase activity at 24 h was 4.6-fold greater than that of WT controls ( $23 \pm 8$  vs.  $5 \pm 4$  U/g;  $P < 0.001$ ;  $n = 7$ ) (Fig. 4B), suggesting significant neutrophil infiltration.

To further investigate lung pathology, we performed H&E staining at 48 h in mice breathing 55%  $O_2$ . We observed the presence of alveolocapillary membrane swelling, inflammatory cell infiltration, extensive capillary disruption, and hemorrhagic extravasation of red cells into alveoli ( $n = 4$ ). These findings were specific to the KO mice breathing 55%  $O_2$  (Fig. 4C).

**Intermittent 11%  $O_2$  Breathing Is Not Effective.** Intermittent hypoxic exposure is currently used in sports training, aviation, and clinical studies of sleep apnea (20–22). Because nighttime hypoxia therapy would allow unhindered patient mobility during the day, we tested whether such a regimen would have a therapeutic effect on KO mice. Starting at 30 d, *Ndufs4* KO mice breathed 11%  $O_2$  for 10 h each day from 9 AM to 7 PM (while sleeping), and 21%  $O_2$  for the remaining 14 h (while awake nocturnally). Daily intermittent exposure to breathing 11%  $O_2$  increased the venous hematocrit to  $56 \pm 4\%$  within 3 wk ( $n = 4$ ) (Fig. 5E), indicating a systemic physiological response to hypoxia. However, the survival time of these mice was not increased relative to that of mice continuously breathing 21%  $O_2$  (58.5 d vs. 58.5 d; log-rank  $P = 0.77$ ; HR, 1.15; 95% CI, 0.45–2.96;  $n = 8$ ) (Fig. 5A). During exposure to intermittent hypoxia, body weight decreased until humane euthanasia criteria were eventually met. Of note, KO mice undergoing the intermittent hypoxic protocol had a significantly lower body weight at 60 d compared with control mice breathing 21%  $O_2$  ( $10.6 \pm 0.8$  g vs.  $12.1 \pm 1.1$  g;  $P < 0.05$ ;  $n = 6$ ) (Fig. 5B). Core body temperature at 50 d was low in both groups ( $32.4 \pm 0.9$  °C vs.  $35.0 \pm 1.5$  °C;  $P < 0.06$ ;  $n = 8$ ) (Fig. 5C). We tested the ability of KO mice to remain on an accelerating, rotating rod, and found no significant difference between KO mice after 3 wk of intermittent hypoxia treatment and control KO mice breathing 21%  $O_2$  (Fig. 5D).

We further studied whether brain lesions in the Leigh syndrome model were ameliorated by intermittent 11%  $O_2$  hypoxic breathing. All four MRI scans of KO mice receiving intermittent treatment revealed hyperintense lesions in the brainstem and OB by 60 d, resembling the neurologic lesions seen in mice breathing 21%  $O_2$  at the same age (Fig. 5F). Thus, although intermittent 11%  $O_2$  hypoxia was sufficient to trigger certain aspects of hypoxia adaptation, such as hematocrit elevation, intermittent hypoxia did not prevent the progression of KO neuropathology.

**Milder Hypoxic Breathing Regimens Are Not Effective.** We previously reported the results of KO mice breathing normobaric 11%  $O_2$ , equivalent to the partial pressure of oxygen found at an altitude of 4,500 m. In the present study, we investigated whether chronic exposure to a milder level of hypoxia would be sufficient to prevent encephalopathic disease. Breathing 17%  $O_2$  for 3 wk, equivalent to living at 1,800 m, starting at 30 d of age did not prevent Leigh syndrome in KO mice (Fig. 6A). After 3 wk of treatment, the core temperature of mice breathing 17%  $O_2$  and normoxic controls was



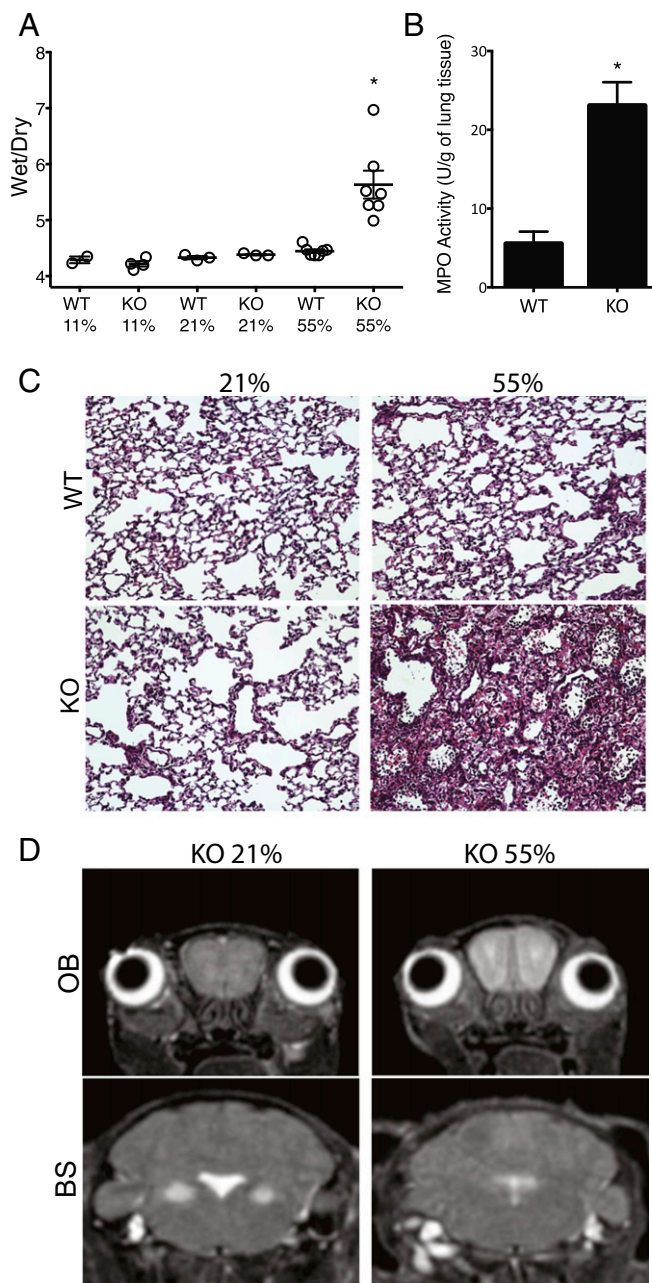
**Fig. 3.** Cardiac ultrasound (US) and cardiac MRI reveal depressed LV myocardial function in *Ndufs4* KO mice breathing 11%  $O_2$  at age >200 d. (A) US of LV fractional shortening (LVFS) of mice breathing various oxygen concentrations at age 200 d ( $n = 6$ ). (B) US LVFS of mice breathing various oxygen concentrations at 50 d of age ( $n = 6$ ). (C) Representative M-mode scans of the left ventricle in WT and KO mice breathing 11%  $O_2$  at 200 d. (D) MRI showing LV ejection fraction of WT and KO mice breathing 11%  $O_2$  at 200 d of age ( $n = 3$ ). (E) 9.4-T MRI showing right ventricular ejection fraction in WT and KO mice breathing 11%  $O_2$  at 200 d of age ( $n = 3$ ). (F) Representative MRI sections of WT and KO mice breathing 11%  $O_2$  at 200 d of age. Images display end-diastolic (ED) and end-systolic (ES) reconstructions. Scans were obtained during sedation with isoflurane while breathing 21%  $O_2$ . Data are mean  $\pm$  SD. \* $P < 0.05$  vs. WT, one-way ANOVA with Bonferroni's correction for multiple comparisons.

reduced to  $35.9 \pm 0.7$  °C and  $35.0 \pm 1.5$  °C, respectively ( $P =$  not significant;  $n = 6$ ) (Fig. 6C). In the same mice, body weight was not significantly changed at 60 d ( $12.5 \pm 1.3$  g vs.  $12.1 \pm 1.1$  g;  $P = 0.54$ ;  $n = 6$ ) (Fig. 6B). Moreover, all KO mice met the humane euthanasia criteria by 90 d (HR, 0.47; 95% CI, 0.20–1.13; log-rank  $P = 0.07$ , 17% vs. 21%  $O_2$ ;  $n = 6$ ).

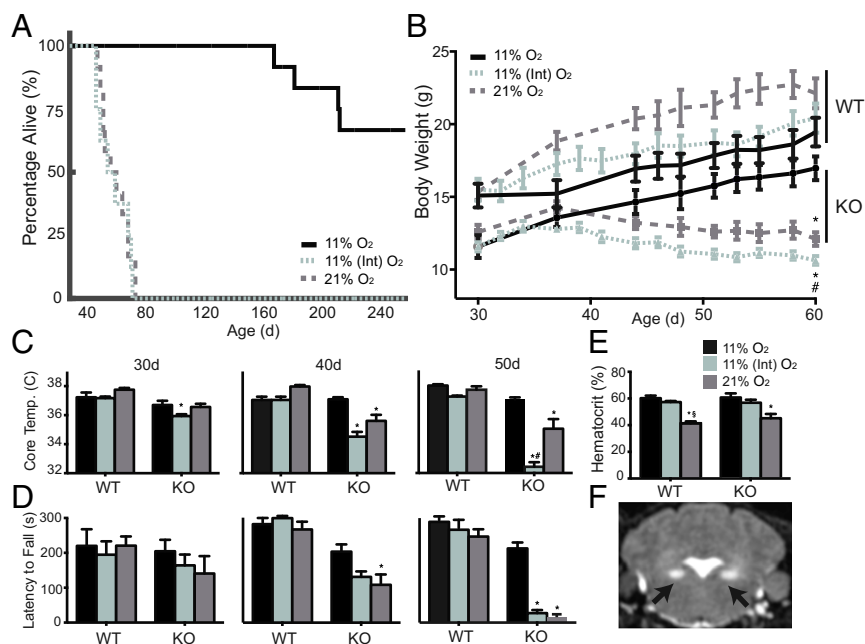
To assess the levels of hypoxemia resulting from changes in inhaled oxygen levels, we measured both murine arterial partial pressure of oxygen ( $PaO_2$ ) and peripheral saturation of oxygen ( $SpO_2$ ) in WT mice. Mice acutely breathing 17%  $O_2$  had a mean  $PaO_2$  of  $77.5 \pm 8$  mmHg and a mean transcutaneous  $SpO_2$  of  $88.5 \pm 3\%$ . Breathing 11%  $O_2$  resulted in a mean  $PaO_2$  of  $45 \pm 4$  mmHg and mean  $SpO_2$  of  $61.3 \pm 2.5\%$  ( $n = 5$ ) (Table 1), resulting in a similar  $PaO_2$  as seen in humans breathing equivalent  $O_2$  pressures (23, 24). After 3 wk of chronic 17%  $O_2$  breathing, the value of venous

hematocrit in KO mice was  $51 \pm 3\%$ , compared with  $60 \pm 6\%$  at 11%  $O_2$  (normoxic control level,  $44 \pm 6\%$ ), ( $P < 0.05$  between groups;  $n = 6$ ) (Fig. 6D). These findings suggest that breathing 17%  $O_2$  triggered an intermediate physiological hematocrit response, but did not prevent the manifestations of neurologic disease.

To test the durability of the effects of hypoxia on neurodegenerative disease, we transitioned three KO mice that were breathing



**Fig. 4.** Detrimental effects of breathing 55%  $O_2$  on lungs and OB of *Ndufs4* KO mice. (A) Pulmonary WD ratio of KO and WT mice breathing various oxygen levels. Lungs were weighed after 24 h of breathing 55%  $O_2$  at age 30 d ( $n = 7$ ). (B) Myeloperoxidase activity in the lungs of mice after 24 h of breathing 55%  $O_2$  at age 30 d ( $n = 7$ ). (C) H&E staining of lungs exposed to 55%  $O_2$  for 48 h at age 30 d ( $n = 3$ , representative images; see text for description). (D) Axial MRI scans of murine brains showing the OB (Upper) and brainstem (BS; Lower) of KO mice breathing 21%  $O_2$  at 60 d and *Ndufs4* KO mice breathing 55%  $O_2$  for 24 h at 30 d (representative images;  $n = 3$ ). Data are mean  $\pm$  SD. \* $P < 0.05$  vs. other groups, one-way ANOVA with Bonferroni's correction.



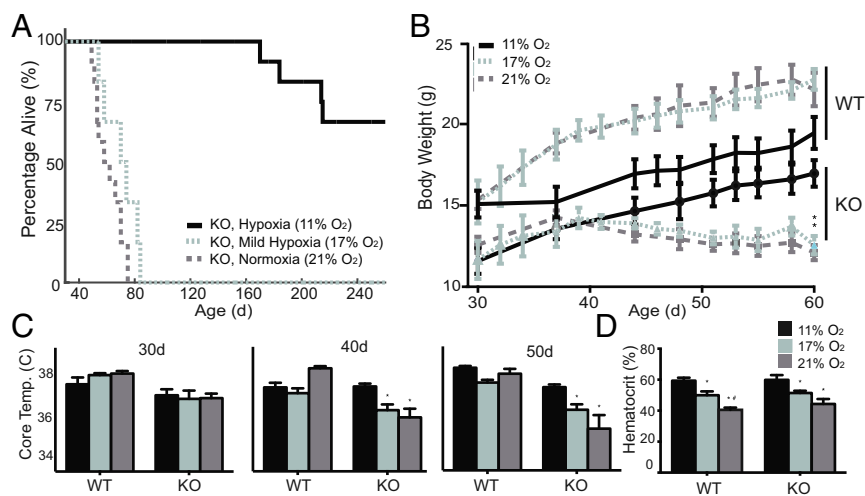
**Fig. 5.** Intermittent hypoxic (11%) breathing (10 h/d) does not alleviate mitochondrial disease in *Ndufs4* KO mice. (A) Survival rates for *Ndufs4* KO mice with intermittent hypoxic breathing (Int) vs. those breathing 21% O<sub>2</sub> (log-rank  $P = 0.77$ ; HR, 1.13; 95% CI, 0.47–2.73;  $n = 8$ ). (B) Body weights after breathing at various oxygen levels and during intermittent hypoxic breathing starting at age 30 d ( $n = 8$ ). (C and D) Core temperature (C) and falling latency (D) from an accelerating, rotating rod for KO mice breathing various oxygen levels or subjected to intermittent hypoxic breathing starting at 30 d of age ( $n = 8$ ). (E) Hematocrit levels in WT and KO mice following 3 wk of exposure to normoxic, hypoxic, or intermittent hypoxic breathing ( $n = 4$ ). (F) Representative MRI in a 60-d-old KO mouse exposed to intermittent hypoxic breathing. Arrows denote lesions in vestibular nuclei. Data are mean  $\pm$  SD. \* $P < 0.05$  vs. KO breathing 11% O<sub>2</sub>; # $P < 0.05$  vs. KO breathing 21% O<sub>2</sub>.

11% O<sub>2</sub> for >100 d back to breathing 21% O<sub>2</sub> air. These KO mice lost weight and died within 4, 5, and 9 d after transitioning to normoxia. This finding highlights the importance of maintaining hypoxic exposure throughout the lifetime of the murine disease model.

#### Breathing 11% O<sub>2</sub> Reverses Clinical Manifestations of Leigh Syndrome.

Patients with Leigh syndrome are typically diagnosed after an acute metabolic crisis, with the concomitant presence of bilateral

lesions detectable by brain MRI (25). Thus, the degenerative brain disease usually manifests before diagnosis. We previously demonstrated that starting hypoxic breathing at an early age (30 d) can prevent the onset of neuropathological disease in the KO mouse model (13). An even more clinically relevant question is whether neuropathological disease can be reversed after its onset. To assess this possibility, we initiated therapeutic hypoxia exposure just before death in untreated KO mice breathing 21% O<sub>2</sub> (55 d). We randomly assigned the KO mice to one of three treatment groups:



**Fig. 6.** Breathing at moderate hypoxia (17% O<sub>2</sub>) does not alleviate mitochondrial disease. (A) Survival rates for *Ndufs4* mice breathing various oxygen levels starting at age 30 d. (B and C) Time course of body weight (B) and body temperature (C) for mice breathing 17% O<sub>2</sub> and those breathing 11% or 21% O<sub>2</sub> for 30, 40, and 50 d ( $n = 6$ ). (D) Venous hematocrit values after 3 wk of exposure to various oxygen levels ( $n = 6$ ). Data are mean  $\pm$  SD. \* $P < 0.05$  vs. 11% oxygen, # $P < 0.05$  vs. 17% oxygen.

**Table 1. SpO<sub>2</sub>, SaO<sub>2</sub>, and PaO<sub>2</sub> values in healthy adult mice and adult human subjects breathing at F<sub>i</sub>O<sub>2</sub> of 11%, 17%, 21%, and 55%**

Variable	F <sub>i</sub> O <sub>2</sub>			
	11%	17%	21%	55%
Mouse SpO <sub>2</sub> , %	61.3 ± 2.5	88.5 ± 3.1	96.8 ± 1.6	99.8 ± 0.3
Mouse SaO <sub>2</sub> , %	60.4 ± 8.1	85.4 ± 2.8	92.3 ± 1.1	100 ± 0
Mouse PaO <sub>2</sub> , mmHg	45 ± 4	77 ± 8	111 ± 14	318 ± 20
Human SaO <sub>2</sub> , %*	83 ± 5	93 ± 2	98 ± 1	100 ± 0
Human PaO <sub>2</sub> , mmHg*	47 ± 8	71 ± 12	110 ± 3	299 ± 4

SpO<sub>2</sub> and SaO<sub>2</sub> in mice were measured using two different techniques (Methods) *n* = 5.

\*Human data from refs. 23, 24.

group A, starting 11% O<sub>2</sub> breathing (hypoxia) at 30 d; group B, starting 11% O<sub>2</sub> breathing at 55 d; and group C, 21% O<sub>2</sub> normoxic breathing controls (Table 2). Mice in all three cohorts appeared healthy at 30 d. By age 55 d, the average body weight was 16.1 ± 3.7 g in group A, 11.6 ± 1.1 in group B, and 11.6 ± 0.9 g in group C (*P* < 0.05, group A vs. group B and group A vs. group C; *n* = 8). Mice in group A demonstrated an upward growth curve trajectory, whereas those in groups B and C exhibited an identical decrease in body weight (*P* < 0.05, group A vs. group B and group A vs. group C) (Fig. 7A). At 60 d of age, the mice in group B were on the fifth day of breathing 11% O<sub>2</sub> and showed an upward weight gain trajectory, whereas the mice in group C continued to lose weight rapidly. By 100 d, the average body weight was 17.7 ± 2.5 g in group A and 16.5 ± 1.9 g in group B (*P* = 0.33; *n* = 8).

In group B mice, average rectal temperature was 33.5 ± 1.2 °C at 5 d before and 35.3 ± 0.9 °C at 5 d after the initiation of 11% O<sub>2</sub> breathing (*P* < 0.01; *n* = 8). Temperature remained stable at subsequent time points (Fig. 7B). Mice in group B could run on an accelerating, rotating rod for 38 ± 23 s at 50 d of age. At age 100 d, endurance on the rotating rod had partially recovered, to 82 ± 73 s (*P* < 0.05; *n* = 9) (Fig. 7C). Ultimately, >70% of the mice in this group were alive at 210 d, as opposed to a median survival of 55 d in group C (HR, 9.7; 95% CI, 3.1–30.1; log-rank *P* < 0.001, *n* = 13) (Fig. 7D). Furthermore, we obtained plasma samples at 40, 50, 60, and 70 d of age from mice in group B. Two previously reported Leigh disease biomarkers (13, 14), α-hydroxybutyrate and lactate, were progressively elevated in normoxia with neurodegenerative disease and decreased after 5 d or 15 d of 11% O<sub>2</sub> breathing (*t* test *P* < 0.05, 50 d vs. 70 d; *n* = 5–8 per group) (Fig. 7E and F). Thus, treating diseased mice with hypoxic breathing after they manifest neurologic disease at 55 d leads to weight gain, increased core temperature, and improved endurance and stability running on a rotating rod, with a significantly increased duration of survival.

**Breathing 11% O<sub>2</sub> Reverses Brain Lesions in Ndufs4 KO Mice with Late-Stage Disease.** Patients with Leigh syndrome develop symmetric, bilateral neurologic lesions, leading to respiratory failure during

childhood. A similar mode of pathogenesis has been demonstrated in KO mice breathing 21% O<sub>2</sub>, with disturbances in the normal respiratory pattern causing death due to respiratory failure between 55 d and 70 d (11, 12). To determine whether the rescue of lifespan and behavior are accompanied by a reversal of the brain lesions, we performed sequential brain MRI scans in four KO mice that had developed the encephalopathic disease breathing 21% O<sub>2</sub> and then breathed 11% O<sub>2</sub> after developing late-stage neurologic disease (treatment group B; Table 2). After their first MRI brain scan, at 55 d, the mice commenced breathing 11% O<sub>2</sub>. Brain MRI was repeated after 2 wk and again after 4 wk of breathing 11% O<sub>2</sub> (Fig. 8). Neuroimaging showed progressive reductions in the intensity and size of lesions in the brainstem and OB after the first 2 wk of hypoxic breathing. In mice with late-stage brain disease, the fourth ventricle appeared more diffuse, likely due to parenchymal atrophy. This abnormal morphology was reversed on breathing 11% O<sub>2</sub>. The aforementioned disease pattern was reversed in all four mice studied.

Finally, to verify our neuroradiologic results, we assessed Iba-1 staining in the same mice treated for >150 d. In these mice, the inflammatory response was no longer present, and hypoxia-rescued brains appeared histologically similar to WT controls (Fig. 9). Future studies will investigate the biochemical nature of pathology reversal. The ability of hypoxia to reverse neurologic lesions may have significant implications for clinical applications, as well as for our understanding of the rescue mechanism.

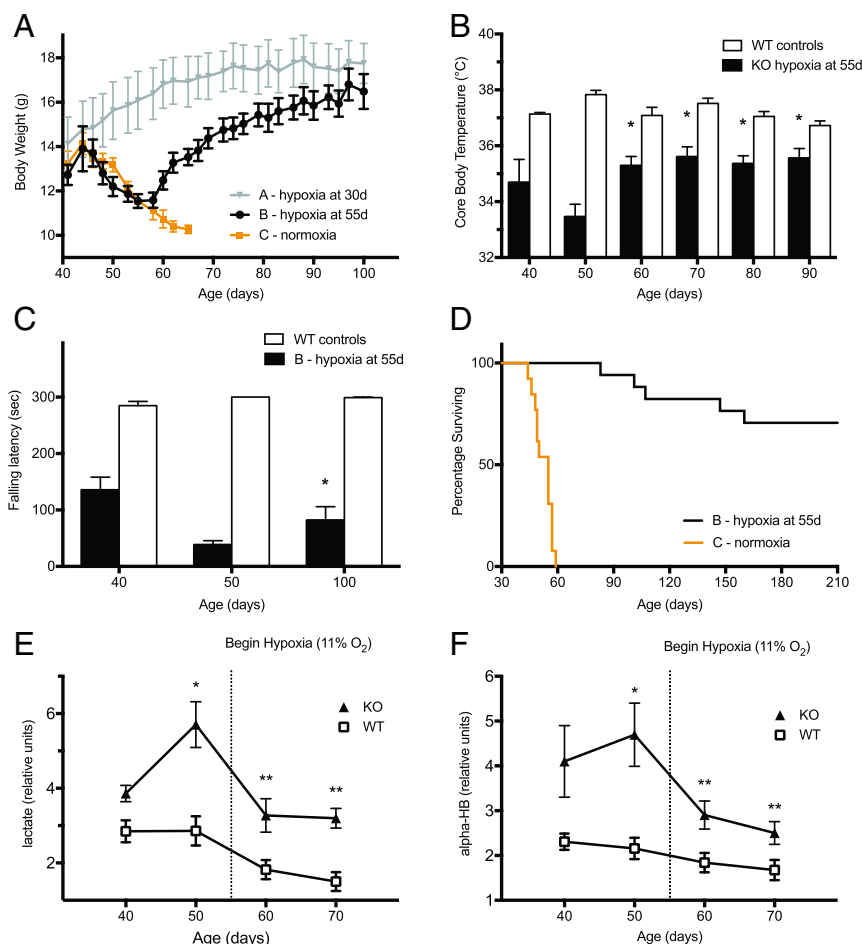
## Discussion

We previously demonstrated that early-onset chronic exposure of the Ndufs4 KO mouse model of Leigh syndrome to breathing 11% O<sub>2</sub> prevented the onset of neurodegenerative disease and extended the duration of KO survival. The goal of our present study was to explore the disease course and pathophysiology of these mice when exposed to breathing different oxygen tensions, and to determine whether alternative hypoxia exposure regimens could be useful. To our knowledge, no other therapeutic approach has demonstrated such high levels of efficacy in mouse models of mitochondrial encephalopathy.

We previously reported that hypoxia extends, and hyperoxia reduces, the lifespan of mice with Ndufs4 deficiency, but eventual lifespan and organ pathologies were not yet known. Here we have shown that whereas Ndufs4 KO mice breathing 21% O<sub>2</sub> develop subacute neurodegeneration, those breathing 11% O<sub>2</sub> do not show signs of neurodegeneration at age >200 d, but rather develop mild LV dysfunction and eventually die at a median age of 270 d. Previous studies have shown that cardiac-specific Ndufs4 KO mice breathing 21% O<sub>2</sub> developed mild cardiac dysfunction (26, 27). Although we cannot conclusively define the cause of death in our hypoxia-treated KO mice, we did observe cardiac dysfunction at age >200 d. In contrast, untreated KO mice breathing 21% O<sub>2</sub> did not exhibit cardiac dysfunction at 50 d, just before their death. One interpretation of these findings is that hypoxic breathing at 11% O<sub>2</sub> is able to prevent neurodegeneration, but not eventual cardiac dysfunction.

**Table 2. Experimental Ndufs4 cohorts exposed to 11% O<sub>2</sub> breathing at different time points**

Group	Genotype	Age at start of 11% O <sub>2</sub> breathing, d	Description of group
A	Ndufs4 <sup>-/-</sup>	30	Breathing 11% O <sub>2</sub> begins before disease onset. Behavior, temperature, and neurologic findings are indistinguishable from those seen in WT mice at this stage.
B	Ndufs4 <sup>-/-</sup>	55	Breathing 11% O <sub>2</sub> begins after disease onset. KO mice have already lost body weight and body temperature and have neurologic lesions detectable by MRI.
C	Ndufs4 <sup>-/-</sup>	—	Normoxic Ndufs4 KO controls.



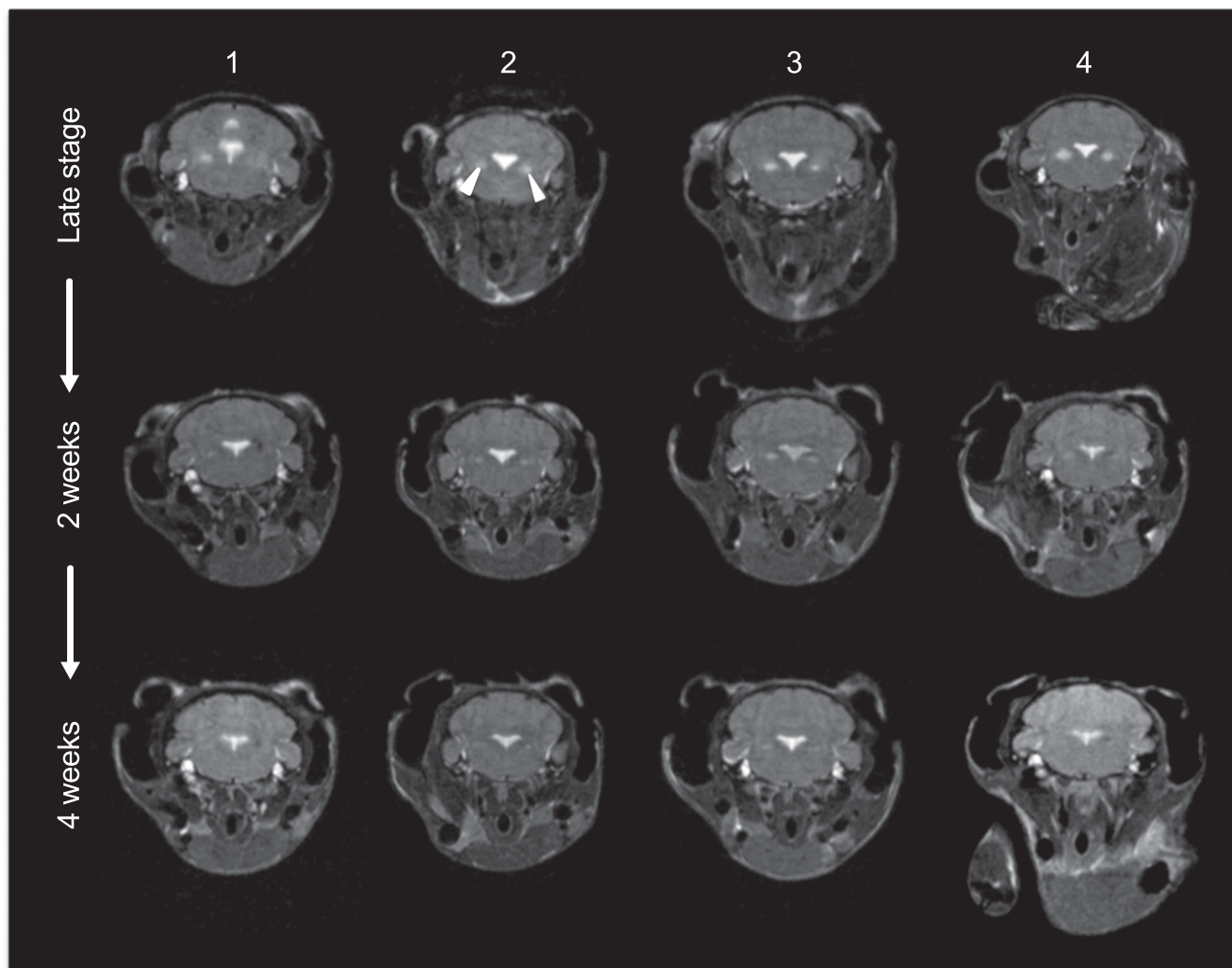
**Fig. 7.** Hypoxic breathing (11%  $O_2$ ) rescues survival, body weight, and behavior of *Ndufs4* mice with late-stage neurologic impairment. (A) Growth curves of *Ndufs4* KO mice exposed to early hypoxic breathing (group A) starting at 30 d of age (gray triangles), those exposed to late hypoxic breathing (group B) starting at 55 d of age (black circles), and control *Ndufs4* KO mice with normoxic breathing (group C; orange squares) (B and C) Body temperature (B) and latency of falling from an accelerating rotating rod (C) in *Ndufs4* KO mice with late-stage disease (group B) and WT controls exposed to breathing 11%  $O_2$  starting at 55 d of age ( $n = 8$ ). (D) Survival rates of hypoxic breathing mice with late-stage disease treated with hypoxia at age 55 d (group B; black,  $n = 17$ ) and controls breathing air (group C; orange,  $n = 13$ ) (log-rank  $P < 0.0001$ ). Data are mean  $\pm$  SD. \* $P < 0.05$  vs. 50 d. (E and F) Lactate (E) and  $\alpha$ -hydroxybutyrate (F) time course in group B mice and WT controls. \* $P < 0.05$ , \*\* $P > 0.05$  compared with WT at 40 d.

We found that breathing 55%  $O_2$  resulted in rapid death of *Ndufs4* KO mice from acute pulmonary edema resulting from acute pulmonary  $O_2$  toxicity, with no MRI evidence of brainstem lesions that were responsible for the death of the untreated mice. The lungs of WT mice exposed to breathing similar  $O_2$  levels for similar periods were normal. This observation is consistent with hyperoxic pulmonary pathology in WT mice at much higher  $O_2$  levels (e.g., 100%  $FiO_2$ ) or even at hyperbaric oxygen pressures (18). It is notable that the respiratory epithelium and the primary olfactory neurons are in direct contact with extremely high inhaled oxygen tensions (not requiring blood oxygen delivery). Our pathophysiological findings highlight the variable tissue-specific oxygen sensitivities of the nose, lung, brain, and heart of *Ndufs4* KO mice with diffusely impaired oxidative metabolism across all of their tissues. We speculate that these differences arise from variations in metabolic flexibility, such as the ability to rely on glycolytic ATP production (28–30), or, alternatively, differences in the ability of these tissues to compensate for oxidative stress (31).

Perhaps the most exciting finding of the present study is that hypoxia can reverse brain lesions of established Leigh syndrome. Most mitochondrial disorders are diagnosed after the first metabolic crisis, and often it is the T2-intense lesions detected on brain MRI that are indicative of mitochondrial disease and help define

the diagnosis. Of note, inherited deficiencies of vitamins, such as thiamine, riboflavin, and biotin, also can lead to Leigh syndrome. Such a deficiency in a mitochondrial metabolite transporter is one of the few truly treatable forms of Leigh disease (5, 6). Our finding that body weight, body temperature, behavior, and even neuro-radiographic and histopathological lesions can be reversed within a few weeks by 11%  $O_2$  breathing offers hope more generally for diverse genetic etiologies of established Leigh disease. We hypothesize two means by which this could take place: (i) either hypoxia is removing the proximal cause of the damage, and thus endogenous repair mechanisms can occur, or (ii) hypoxia actively triggers repair mechanisms that restore damaged tissue. Cardiomyocytes are known to be capable of regeneration and proliferation when exposed to very low oxygen levels even after completion of fetal development (32). Furthermore, hypoxia recently has been shown to improve adult neurogenesis (33–35).

An important question is whether the doses of hypoxia used in mice in the present study can be extrapolated to humans. We are optimistic about this prospect. Our healthy hypoxic mice breathing 11% oxygen had an average  $PaO_2$  of 45 mmHg (Table 1). Human volunteers at the Mont Blanc research station (4,559 m), equivalent to breathing 11% oxygen at sea level, had an average  $PaO_2$  of 46 mmHg (36). Of course, the ability of hemoglobin to bind  $O_2$  is



**Fig. 8.** Breathing 11%  $O_2$  in late-stage neurological disease reverses the radiographic lesions of *Ndufs4* mice seen on MRI. Four *Ndufs4* mice were breathing 21%  $O_2$  until they developed late-stage neurological disease (55 d). They underwent MRI to document bilateral lesions in the vestibular nuclei (*Upper*, white arrow). Subsequently they commenced breathing 11% oxygen. The mice were scanned again at 2 wk and 4 wk of hypoxic breathing (*Middle* and *Lower*, respectively). Neurologic lesions disappeared by 4 wk of hypoxic breathing. The section of the fourth ventricle shown at the center of the brainstem appeared enlarged in the early scans, suggesting parenchymal atrophy. After 4 wk of treatment, this area appeared to be reduced in size, and morphological relationships were restored.

lower in mice than in humans, which is why murine arterial oxygen saturation ( $SaO_2$ ) is lower despite a comparable  $PaO_2$  (Table 1). Nonetheless, if  $PaO_2$  is the key parameter in alleviating mitochondrial pathology, an 11%  $FiO_2$  in humans ought to be comparable to that in mice. Furthermore, in murine models of chronic hypoxia (37), breathing 11% oxygen at sea level produces pulmonary hypertension like that seen in humans, suggesting that the pulmonary vasoconstrictor response to breathing 11% oxygen is conserved across both species. Thus, several major aspects of oxygen transport and physiology show comparable dose responses in humans and mice.

Although chronic, continuous 11%  $O_2$  breathing has a dramatic effect in *Ndufs4* KO mice, more practical regimens are desirable. In the present study, however, we found that neither more moderate, chronic hypoxic breathing of 17%  $O_2$  nor intermittent 11%  $O_2$  breathing for a duration of 10 h each day was effective. We also found that the therapeutic effects of chronic hypoxia in mice do not appear to be durable; when hypoxia-treated mice were returned to normoxic breathing, they died after 1 wk of air breathing. Thus, the only currently available regimen that prevents

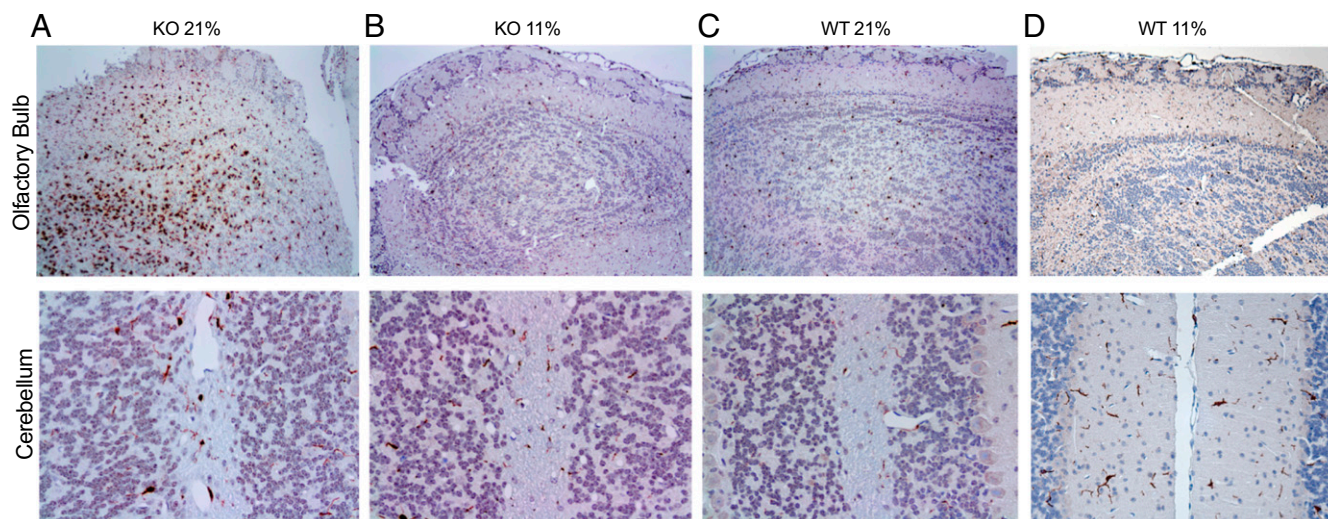
and reverses Leigh syndrome in *Ndufs4* KO mice is continuously breathing 11%  $O_2$ .

Although the *Ndufs4* KO mouse is perhaps the most accurate mouse model of Leigh syndrome, its disease trajectory is distinct from that of humans. In particular, this mouse exhibits very uniform, downward trajectories of body temperature, body weight, and activity, typically dying at around day 55. The disease course in humans tends to be more variable and episodic, and in fact, more practical hypoxia regimens may be intermittently effective in helping repair neurodegenerative lesions in humans. Future investigations are needed to determine whether hypoxia can be delivered safely and effectively as therapy for mitochondrial diseases.

## Methods

**Animal Care.** *Ndufs4*<sup>+/-</sup> mice were generously provided by the Palmiter laboratory at the University of Washington. We continuously bred heterozygous mice to provide sufficient *Ndufs4*<sup>-/-</sup> and control (*Ndufs4*<sup>+/-</sup> and WT) mice for our experiments. Because *Ndufs4*<sup>+/-</sup> and WT mice are identical in all reported assays performed by us and others, we treated both groups as controls. Pups were weaned and genotyped at ~25 d after birth. All cages were provided with daily food, water, and hydrated gel (Hydrogel). Food





**Fig. 9.** Breathing 11% O<sub>2</sub> in late-stage neurological disease reverses pathological inflammation in the brains of *Ndufs4* KO mice. Representative images with Iba-1 staining of the OB and cerebellum (CB) in KO mice and WT controls ( $n = 3$  per group). Iba-1 is a marker of inflammation in the brain, indicative of microglial activation. Images demonstrate the reversibility of the neuropathological pattern by breathing 11% O<sub>2</sub> at the late stage of disease (55 d). (A) KO mice breathing 21% O<sub>2</sub>. (B) KO mice breathing 21% O<sub>2</sub> up to 55 d and then breathing 11% O<sub>2</sub> (to 160 d). MRI-demonstrated reversal of the lesions reported in Fig. 8 was observed in these same mice. (C) Normoxic WT controls. (D) Hypoxic WT controls.

and gel were replaced three times per week, and cages were changed weekly (additional food was placed on the bedding). Body weights were recorded regularly, and mice were humanely euthanized when they had lost 20% of peak body weight, in accordance with the most recent American Veterinary Medical Association guidelines. Animals were randomized on a 1:1 basis, balanced by age and sex. All animal studies were approved by the Subcommittee on Research Animal Care and the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

**Hypoxic Chambers.** Mice were housed in 80-L transparent acrylic boxes. The desired FiO<sub>2</sub> was obtained by mixing a constant flow of medical air with pure nitrogen from a liquid nitrogen tank (Airgas) or by direct separation of nitrogen and oxygen present in room air by a nitrogen generator (MAG-20; Higher Peak). The total gas flow through the chamber was measured using a flowmeter (Cole Parmer) and adjusted to 5–10 L/min to maintain chamber CO<sub>2</sub> concentrations below 0.4% (CO200; Extech). Soda lime was added to the chambers as a CO<sub>2</sub> scavenger. The oxygen concentration was measured at the outlet port of the chamber with an O<sub>2</sub> sensor (MiniOx 1; Ohio Medical), which was calibrated weekly using an 8.55% O<sub>2</sub> reference tank (Airgas). Oxygen levels inside the chambers were tolerated within a 0.4% offset from the target concentration by adjusting nitrogen flow as needed. Temperature was maintained at 24–26 °C, and humidity was maintained at 30–70%. A standard light-dark cycle of ~12h light exposure was used. Mice were housed in cages with standard bedding and given unlimited access to food and water.

**MRI.** MRI scans of the brain were performed with the mice under general anesthesia with isoflurane 0.5–1.5% in 21% O<sub>2</sub>. T2-weighted RARE (rapid acquisition of refocused echoes) MRI images were acquired on a 4.7-T small animal scanner (Pharmascan; Bruker) with a transmit-receive volume coil and the following parameters: repetition time (TR), 6,000 ms; echo time (TE), 60 ms; rare factor, 10; 24 slices; 192 × 192 matrix; 0.130 × 0.130 × 0.7 mm voxels; eight averages. The data were converted from the RAW Bruker format into DICOM images and visualized with a freeware DICOM reader (Osirix; University of Geneva).

Cardiac MRI was performed with a 9.4-T horizontal-bore small animal MRI scanner (Biospec; Bruker) equipped with a 1,500-mT/m gradient system. Images were acquired with an MRI-compatible cardiorespiratory gating system (SA Instruments) and a transmit-receive surface coil. Cine imaging was performed on the short axis of the heart from base to apex with a gradient echo cine sequence and the following parameters: field of view, 25 × 25 mm<sup>2</sup>; slice thickness, 1 mm with no gaps; matrix, 200 × 200; 20 frames per cycle; TR, RR interval/20; TE, 1 ms; flip angle, 30°; four averages. The data were converted in MATLAB (Mathworks) into the DICOM format and analyzed with the Osirix freeware DICOM reader.

**Brain Histology.** Mice were placed under deep anesthesia with ketamine and fentanyl, and a needle was inserted into the left ventricle. The whole body was perfused with ice-cold PBS, followed immediately by 4% PFA. The brain was removed, stored in 4% PFA for 24–48 h, and then placed in 30% sucrose (in PBS) for 48 h. Fixed brains were sectioned parasagittally. In addition, two transverse sections of cerebellum were prepared: a rostral section with subjacent pons and a more caudal section with medulla oblongata. Immunohistochemistry was performed using an antibody against the microglial marker Iba-1 (Wako) at 2 μg/mL.

**Echocardiography.** For LV studies, mice were scanned using a 13-MHz probe (Vivid 7; GE Healthcare). All scans were performed by an experienced echocardiographer who was blinded to the study group assignments. During the procedure, all mice were breathing 21% O<sub>2</sub> and were anesthetized with isoflurane (0.5–1.5%) titrated to maintain a heart rate between 500 and 600 bpm.

**Pulmonary Studies.** The WD ratio was calculated by dividing the wet lung tissue weight by the dry lung tissue weight. Organs were left to dry for 24 h at 60 °C to obtain dry lung tissue. Frozen lung samples were stored at –80 °C, and myeloperoxidase activity was assessed as described previously (38). Lungs were perfused for H&E staining using a wash solution of Ringer's lactate followed by 4% PFA. Perfusions were performed during deep anesthesia, followed by open chest puncture of the cardiac apex using a 20-gauge needle. A 1-mm incision was made in the right atrium to allow outflow of blood from the venous return. After 1 min, the lungs were inflated with 4% PFA through the trachea, allowing 1 min for proper filling of alveoli. A pressure of ~30 cmH<sub>2</sub>O was obtained by gravity flow. Organs were stored overnight in PFA and processed on slides by the Histopathology Core at Massachusetts General Hospital.

**Behavior.** Behavioral experiments were performed using a Rotarod (Ugo Basile) machine with and acceleration of 5 rpm/min and a maximum speed of 40 rpm. Mice were placed on the accelerating, rotating rod while breathing room air. Latency to fall was measured up to a maximum time of 300 s. The test was performed three times, with a minimum of 10 min between sessions to allow for recovery. The median latency to fall was reported to avoid the incorporation of aberrant behavior trials. If mice latched onto a rotarod rather than walking on the rod for more than 10 s, this time was recorded as the latency to fall.

**Biomarker Measurements.** Isotope-labeled standards (CDN isotope) of lactate and  $\alpha$ -hydroxybutyrate were used to generate a standard curve. This allowed for absolute quantification of metabolites in mouse plasma. For this, 30 μL of the mouse plasma sample was combined with 20 μL of isotope-labeled internal standard, and 70% acetonitrile was used for metabolite extraction. LC-MS was

performed on a Q Exactive Plus Orbitrap mass spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific). The Xbridge amide HILIC column (2.1 × 100 mm, 2.5 μM particle size; Waters, 186006091) was used for separate tests in the negative ionization mode. Mobile phase A was 20 mM ammonium acetate and 0.25% ammonium hydroxide (pH 9). Mobile phase B was 100% acetonitrile. Data acquisition was performed in full scan mode, selected for a range of 70–1,000 *m/z*, with resolution of 140,000, an automatic gain control target of 3e6, and a maximum injection time of 400 ms.

**Blood and Tissue Collection.** For collection of plasma and tissues, mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and fentanyl (0.09 mg/kg). After tracheostomy, volume-controlled ventilation was maintained at a respiratory rate of 90 breaths/min, a tidal volume of 10 ml/kg, a positive end-expiratory pressure of 1 cmH<sub>2</sub>O, and an inspired O<sub>2</sub> of 21% (Mini Vent 845; Harvard Apparatus). Whole blood was collected by cardiac puncture and placed in an EDTA-containing tube. Plasma was obtained by centrifuging whole blood at 2,000 × *g* for ~20 min. Tissues were harvested and immediately flash-frozen in liquid nitrogen. When repeat samples were required, a small tail nick was performed, and blood was collected in heparinized glass capillary tubes. Blood samples were then centrifuged as described above.

**Transcutaneous Awake SpO<sub>2</sub> and Arterial Blood Gas Tensions at Various FIO<sub>2</sub> Values.** Healthy, unanesthetized adult C57Bl6 mice were placed inside a 5-L chamber, and gas flow was set to maintain chamber CO<sub>2</sub> concentration below 0.1% (CO200, Extech). Oxygen concentrations were measured at the chamber inlet and outlet using an O<sub>2</sub> analyzer (MiniOx 1). A murine transcutaneous oximeter collar probe was placed on the mice to allow for unrestrained measurements (MouseOx Plus; Starr Life Sciences). The chamber F<sub>I</sub>O<sub>2</sub> was adjusted to obtain 11%, 17%, and 21% O<sub>2</sub> sequentially. The mice were allowed to adapt for 5 min at each oxygen level. SpO<sub>2</sub> values were recorded continuously for the next minute and averaged.

For arterial measurements, additional mice were anesthetized with sevoflurane 3.5%. A PE-10 catheter was used to cannulate the carotid artery after ligation. Mice were allowed to recover for 1 h after surgery. At the end of the procedure, mice were placed in a 5-L acrylic chamber that was constantly ventilated with the desired FIO<sub>2</sub>. Mice were allowed a 5-min adaptation to each FIO<sub>2</sub> level (randomized sequence), after which heparinized arterial blood was sampled and analyzed with a blood gas analyzer (ABL800; Radiometer).

**Statistics.** Data are reported as mean ± SD. Analyses were performed using GraphPad Prism 6.0 software. The two-sample Student *t* test was used for two-group comparisons. One-way ANOVA with Bonferroni's correction was used for multiple comparisons. The log-rank test was performed, and HRs with 95% CIs were calculated to compare survival rates. A *P* value < 0.05 was considered to indicate statistical significance.

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