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Genetically altering organismal metabolism by leptin-deficiency benefits a mouse model of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal, neurodegenerative disease that causes death of motor neurons. ALS patients and mouse models of familial ALS display organismal level metabolic dysfunction, which includes increased energy expenditure despite decreased lean mass. The pathophysiological relevance of abnormal energy homeostasis to motor neuron disease remains unclear. Leptin is an adipocyte-derived hormone that regulates whole-animal energy expenditure. Here, we report that placing mutant superoxide dismutase 1 (SOD1) mice in a leptin-deficient background improves energy homeostasis and slows disease progression. Leptin-deficient mutant SOD1 mice possess increased bodyweight and fat mass, as well as decreased energy expenditure. These observations coincide with enhanced survival, improved strength and decreased motor neuron loss. These results suggest that altering whole-body energy metabolism in mutant SOD1 mice can mitigate disease progression. We propose that manipulations that increase fat mass and reduce energy expenditure will be beneficial in the setting of motor neuron disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease in which death of both upper and lower motor neurons leads to progressive weakness and death. Disease pathogenesis is linked to mitochondrial dysfunction, oxidative stress, excitotoxicity, perturbation of RNA biology and impairment of proteostasis (1–3).

A growing body of the literature highlights the presence of metabolic perturbations in ALS (4–6). A subset of patients are hypermetabolic, which is defined as $\geq$1.1 ($\geq$10%) of the ratio of measured resting energy expenditure (mREE) to calculated resting energy expenditure (cREE) or mREE/cREE (7). ALS patients show increased resting energy expenditure (+16–18%) and decreased fat free mass (4,7–11). These abnormalities are seen early in disease, are progressive and are present in both sporadic ALS and familial ALS (fALS) patients (7). Similarly, several mouse models of ALS, including transgenics, which overexpress mutant forms of copper zinc superoxide dismutase1 (SOD1), show increased energy expenditure by indirect calorimetry, as well as decreased bodyweight (12,13). Mutant TAR DNA-binding protein 43 (TDP-43) mice have also been reported to exhibit altered organismal metabolism (14,15).

Previous studies have investigated the potential therapeutic impact of dietary manipulations by administering a high-calorie diet to mutant SOD1 mice (5,6,13). These studies report various beneficial effects, including increased survival and improved motor behavior in mutant mice. Because each study utilized different types of high-calorie diets, the extent to which the benefits of these dietary manipulations can be ascribed to provision of a specific nutrient(s) versus simply calories, *per se*, is unknown. Furthermore, whether or not these dietary manipulations correct

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or improve the hypermetabolic phenotype of mutant SOD1 mice have not been reported.

Leptin is an adipocyte-derived hormone that signals to peripheral tissues and the brain to induce satiety and increase energy expenditure (16). Humans and mice that are null for the leptin gene (*ob*) are hypometabolic, hyperphagic and morbidly obese. Leptin-haploinsufficient (*ob/+*) mice possess ~70% of wild-type serum leptin levels and on average, 35% increased adiposity compared with wild-type controls (17,18). *Ob/+* mice are neither obese nor hyperphagic, but are thought to have reduced metabolic rates since they survive periods of fasting longer than control counterparts (19). To test the notion that motor neuron disease could be mitigated by changes in organismal metabolism, we placed mutant SOD1 mice in the leptin-deficient *ob/+* or *ob/ob* backgrounds and investigated changes in body composition, energy expenditure, motor function, and lifespan.

RESULTS

Leptin-deficient, mutant SOD1 animals demonstrate blunted weight and fat loss

To investigate the effects of manipulating organismal metabolism in a motor neuron disease model, we utilized hemizygous mice harboring the G93A mutation, a well-characterized model of ALS (20). G93A mice possess high copy numbers of the human mutant SOD1 transgene, as well as display disease symptoms, such as paralysis and muscle weakness similar to ALS patients. Mutant SOD1 mice were placed in the background of leptin-deficient *ob* animals in a two-step breeding strategy (Fig. 1A). Genotypes were generated at expected frequencies and litter sizes were normal (Supplementary Material, Table S1). We refer to hemizygous G93A mutant SOD1 animals as *SOD1;+/+*, while G93A mutant SOD1 animals on the mutant leptin heterozygote and homozygote backgrounds are denoted as *SOD1;ob/+* and *SOD1;ob/ob*, respectively. Non-transgenic mice are denoted as *+/+;+/+* (leptin wild type), *+/+;ob/+* (mutant leptin heterozygote) and *+/+;ob/ob* (mutant leptin homozygote).

Previous literature has reported decreased adipose tissue and circulating leptin levels in mutant SOD1 animals (13). To assess the interaction between leptin-haploinsufficiency and the SOD1 mutation, serum leptin levels were measured by enzyme-linked immunosorbent assay (ELISA). Male and female leptin-haploinsufficient (*+/+;ob/+*) mice possessed ~50% of wild-type serum leptin levels at two time points, P90 and P120 ($P < 0.05$). There was no difference between *SOD1;ob/+* and *+/+;ob/+* animals (Fig. 1B and C). In male, but not female mice, there was a significant reduction in serum leptin levels in *SOD1;+/+* compared with *+/+;+/+* at both P90 and P120 ($P < 0.05$). These data show that the G93A mutation does not affect circulating leptin levels in a leptin-haploinsufficient background. We also observed sex-specific effects in circulating leptin levels. *SOD1;+/+* male, but not female mice, showed decreased serum leptin levels compared with *+/+;+/+* controls.

To further investigate the effects of leptin deficiency on whole-body metabolism in the mutant SOD1 mice, we recorded bodyweight in both females and males, beginning at P40, an early, asymptomaptic phase of disease until ~P120, a later, symptomatic phase of disease (Fig. 2A and B). Although all mice gained weight over time ($F_{(12,372)} = 62.51$, $P < 0.0001$), there was a significant interaction effect between time and genotype ($F_{(36,372)} = 3.63$, $P < 0.0001$). At P60, an early time point of disease, there were no significant differences in bodyweight between mutant SOD1 animals and their control counterparts, with the exception of *SOD1;+/+* males ($P = 0.02$ compared with *+/+;+/+* males) (Fig. 2A and B; Supplementary Material, Table S2). Bodyweight differences between mutant SOD1 animals and non-Tg counterparts begin to largely diverge around P90. At this time point, *SOD1;+/+* mice weighed, on average, 15% less than *+/+;+/+* animals ($P = 0.02$ and $P < 0.001$ in females and males, respectively), while *SOD1;ob/+* mice weighed ~5% less than *+/+;ob/+* counterparts. By P120, *SOD1;+/+* mice weighed ~18% less than *+/+;+/+* animals ($P < 0.01$ and $P < 0.0001$ in females and males, respectively), while *SOD1;ob/+* mice weighed ~15% less than *+/+;ob/+* animals ($P < 0.001$ and $P < 0.02$ and in females and males, respectively).

However, the degree of weight loss was blunted in the *ob* haploinsufficient background at several time points of disease (P60: $P < 0.02$ for males, P90: $P \leq 0.02$ for females and males). At P120, female *SOD1;ob/+* mice weighed ~7% greater than SOD1;+/+ counterparts ($P \leq 0.03$). Similar to *+/+;ob/ob* controls, *SOD1;ob/ob* female and male mice are also obese and weighed more than twice the average bodyweight of *SOD1;+/+* and *SOD1;ob/+* littermates at all-time points of disease ($P < 0.0001$) (Fig. 2A and B; Supplementary Material, Table S2).

Differences in bodyweight have been attributed to decreased fat mass in mutant SOD1 mouse models (13). Consistent with overall weight loss, both female and male *SOD1;+/+* mice showed a reduction in gonadal fat pads, the largest depot of white adipose tissue (WAT), compared with *+/+;+/+* littermates. This observation was seen at both P90 and P120 ($P \leq 0.05$) (Fig. 2C). Compared with *SOD1;+/+*, *SOD1;ob/+* mice possessed heavier gonadal fat pads ($P < 0.04$ and $P < 0.008$ at P90 and P120, respectively) (Fig. 2C). *SOD1;ob/ob* mice possessed large gonadal fat pads, which weighed five times more than both *SOD1;+/+* and *SOD1;ob/+* animals (data not shown). Furthermore, at a later stage of disease (P150), *SOD1;+/+* mice are grossly cachexic, and this phenotype is attenuated in leptin-deficient *SOD1;ob/+* mice (Fig. 2D). Decreased adiposity in *SOD1;+/+* mice and rescue in *SOD1;ob/+* mice were confirmed by dual-energy X-ray absortiometry (DEXA) scanning. *SOD1;+/+* mice demonstrated a significant decrease in total body WAT content compared with *+/+;+/+* controls at P90 and P120 ($P = 0.047$ and $P < 0.001$, respectively). This loss was attenuated in *SOD1;ob/+* mice ($P < 0.02$ at P90 and P120) (Fig. 2E).

Improved bodyweight in leptin-deficient mice was specific to improvements in WAT. There were no differences among genotypes in lean mass, an indicator of muscle mass, at P90. At P120, *SOD1;+/+* showed reduced lean mass compared with *+/+;+/+* mice ($P = 0.0006$), although this loss was not rescued in *SOD1;ob/+* mice (Fig. 2F). No significant weight differences among genotypes were also observed in brown adipose tissue (BAT) (Fig. 2G). There were also no significant differences in total bone mineral density (BMD) among genotypes, although *SOD1;+/+* animals had decreased abdominal BMD

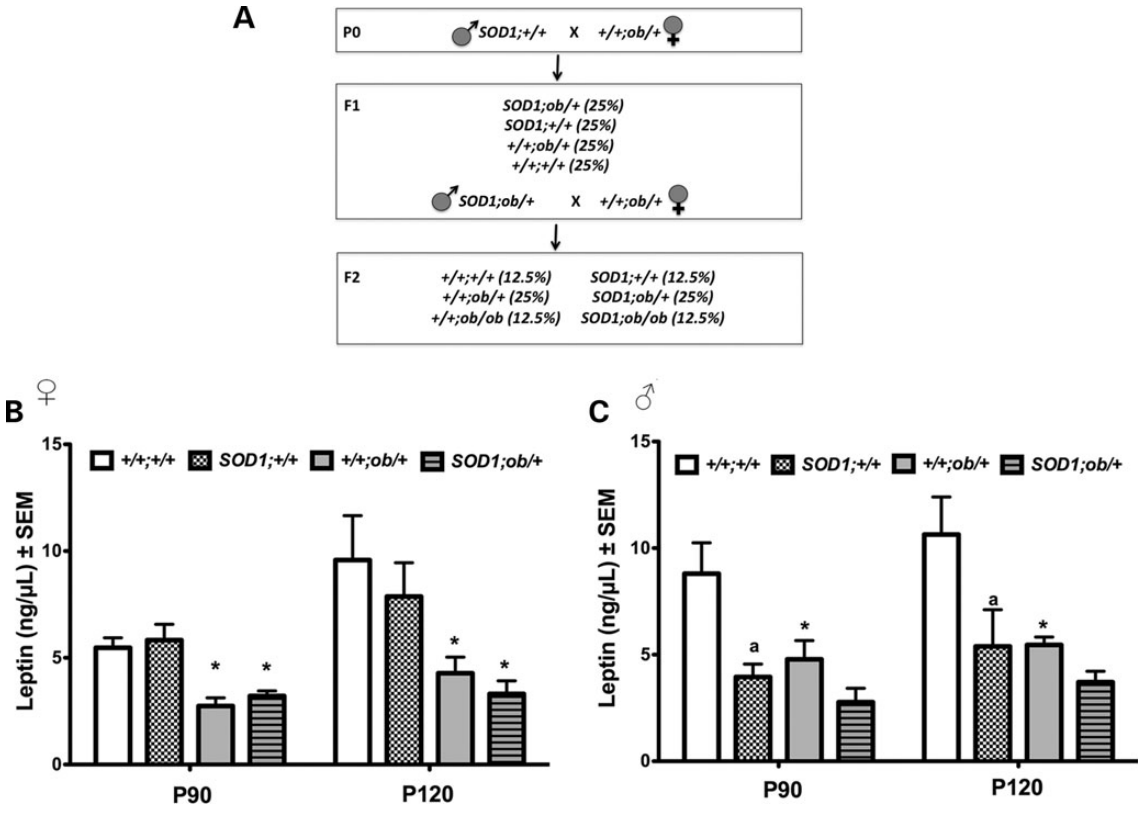

Figure 1. (**A**) Two-step breeding strategy to generate leptin-deficient mutant SOD1 mice. In parenthesis are the expected genotype frequencies. (**B**) Serum leptin levels from P90 and P120 mice. Leptin-haploinsufficient mice possess 50% of wild-type serum leptin levels ($^{*}P < 0.05$). Only *SOD1;+/+* males possess significantly decreased serum leptin levels at both P90 and P120 ($^{a}P < 0.05$). $n \geq 7$. Error bars ± SEM.

compared with *+/+;+/+* mice. Decreased abdominal BMD was not rescued in the *SOD1;ob/+* mice (Fig. 2H).

Abnormal energy expenditure is attenuated in leptin-deficient, mutant SOD1 animals

Are changes in bodyweight and fat mass associated with alterations in overall energy expenditure? ALS patients and mutant SOD1 animals have been reported to be hypermetabolic (7,13). We therefore measured energy expenditure (VO_2) by indirect calorimetry and found a significant effect of genotype on average VO_2 levels at P120 ($F_{(3,32)} = 10.89$, $P < 0.001$). Both female and male *SOD1;+/+* mice possessed significantly elevated VO_2 levels compared with *+/+;+/+* mice (+16%, $P < 0.001$) (Fig. 3A and B). Placing mutant SOD1 mice in a leptin-deficient background blunted this hypermetabolic phenotype. Although *SOD1;ob/+* mice still showed elevated VO_2 levels compared with *+/+;ob/+* controls (+10%, $P < 0.008$), *SOD1;ob/+* animals demonstrated a significant reduction in VO_2 levels compared with *SOD1;+/+* animals ($P = 0.02$). This effect was more pronounced in females compared with males. Although there was a trend for male *SOD1;ob/+* mice to possess lower VO_2 levels compared with *SOD1;+/+* animals, this did not attain statistical significance (Fig. 3B). *SOD1;ob/ob* animals were significantly hypometabolic compared with *SOD1;+/+* and *SOD1;ob/+* littermates ($P < 0.0001$) (Fig. 3A and B). Reduced VO_2 levels in leptin-deficient mice was apparent during both the light ($P < 0.0002$) and dark cycles ($P \leq 0.05$) (Fig. 3C and D). Between *+/+;+/+* and *+/+;ob/+* animals, no significant differences in average, light and dark cycle VO_2 levels were noted (Fig. 3A and B; Supplementary Material, Fig. S1). Leptin null (*+/+;ob/ob*) mice were also significantly hypometabolic compared with *+/+;+/+* and *+/+;ob/+* littermates ($P < 0.0001$) (Supplementary Material, Fig. S1).

The differences in bodyweight, body composition and energy expenditure between *SOD1;+/+* versus *SOD1;ob/+* animals could not be explained by differences in food consumption (Fig. 3E), activity levels (Fig. 3F) or hyperthyroidism (Fig. 3G). In contrast, obesity in leptin null *+/+;ob/ob* and *SOD1;ob/ob* animals was associated with hyperphagia and decreased activity levels ($P < 0.0001$ different from all genotypes for both food intake and activity levels) (Fig. 3E and F).

Gene expression in skeletal muscle and BAT do not correspond with the hypermetabolic phenotype of mutant SOD1 mice

Previous studies have implicated skeletal muscle as a significant contributor to the hypermetabolic phenotype of mutant SOD1 mice (21,22). To gain insight on the beneficial effects of our genetic manipulation, we profiled genes involved in metabolism in two kinds of skeletal muscle. In the soleus muscle, a two-way ANOVA revealed significant main effects of genotype on gene

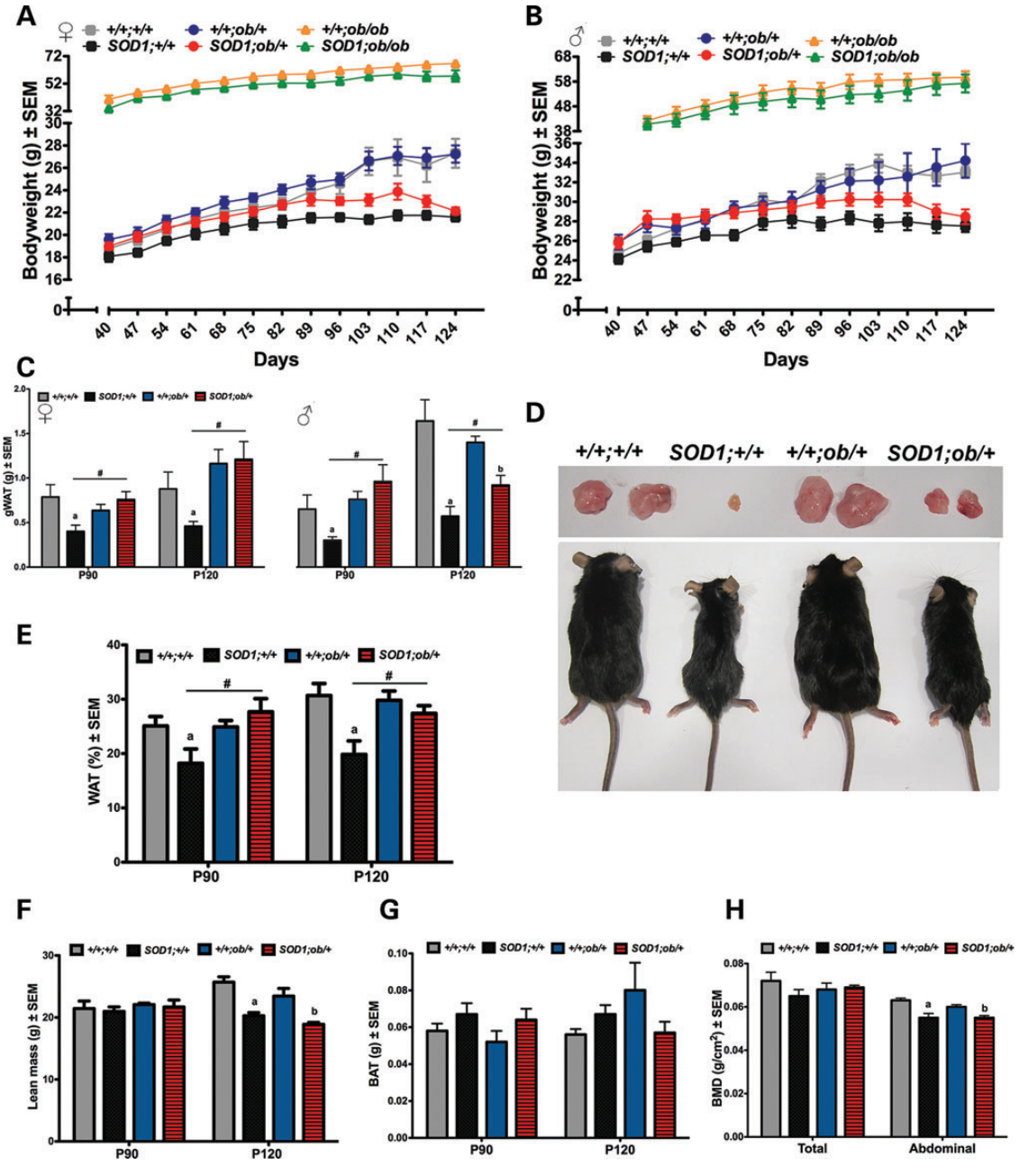

Figure 2. Leptin-deficient, mutant SOD1 mice display improved bodyweight and WAT mass. Female (**A**) and male (**B**) non-Tg mice gain weight between P40 and ∼P120. Although the bodyweight of mutant SOD1 animals start plateauing around P90, leptin-deficient *SOD1;ob/+* and *SOD1;ob/ob* still weigh more compared with *SOD1;+/+* mice. $n \geq 7$. (**C**) *SOD1;+/+* animals have significantly less gonadal WAT (gWAT) than +/+;+/+ mice ($^{a}P \leq 0.05$), which is rescued in *SOD1;ob/+* animals at P90 ($^{\#}P < 0.04$) and P120 ($^{\#}P < 0.008$). $n \geq 5$. (**D**) P150 mice and respective gonadal WAT pads. Cachexia is attenuated in *SOD1;ob/+* mice. (**E**) As measured by DEXA scan, the total % of WAT is less in *SOD1;+/+* compared with +/+;+/+ mice at P90 ($^{a}P = 0.047$) and P120 ($^{a}P < 0.001$). This defect is rescued in *SOD1;ob/+* mice ($^{\#}P < 0.02$). $n \geq 3$. (**F**) Total lean mass is decreased in P120 mutant SOD1 compared with non-Tg mice ($^{a,b}P \leq 0.002$). $n \geq 3$. (**G**) There are no differences in BAT weight among genotypes. $n \geq 5$. (**H**) P120 mutant SOD1 animals possess significantly less abdominal BMD compared with non-Tg controls ($^{a,b}P < 0.04$). $n \geq 3$. Error bars ± SEM. [a]Significantly different from +/+;/+/+, [b]significantly different from +/+;*ob*/+, [#]significantly different from *SOD1;*/+/+.

expression ($F_{(3,101)} = 23.72$, $P < 0.0010$) (Fig. 4A). Genes involved in fatty acid oxidation (FAO), such as CPT1b, MCAD and LCAD, as well as electron transport chain (ETC) activity, such as ATP5g1, Cytc, Ndufa2 and COXIV, were significantly decreased (or showed a trend towards reduced expression) in *SOD1;+/+* compared with +/+;+/+ animals. Partial rescue of mRNA levels back to control levels was also observed in *SOD1;ob/+* animals for several of these genes, but this did not reach significance. In the gastrocnemius (gastroc) muscle of *SOD1;+/+* mice, CPT1b and UCP3 levels were significantly

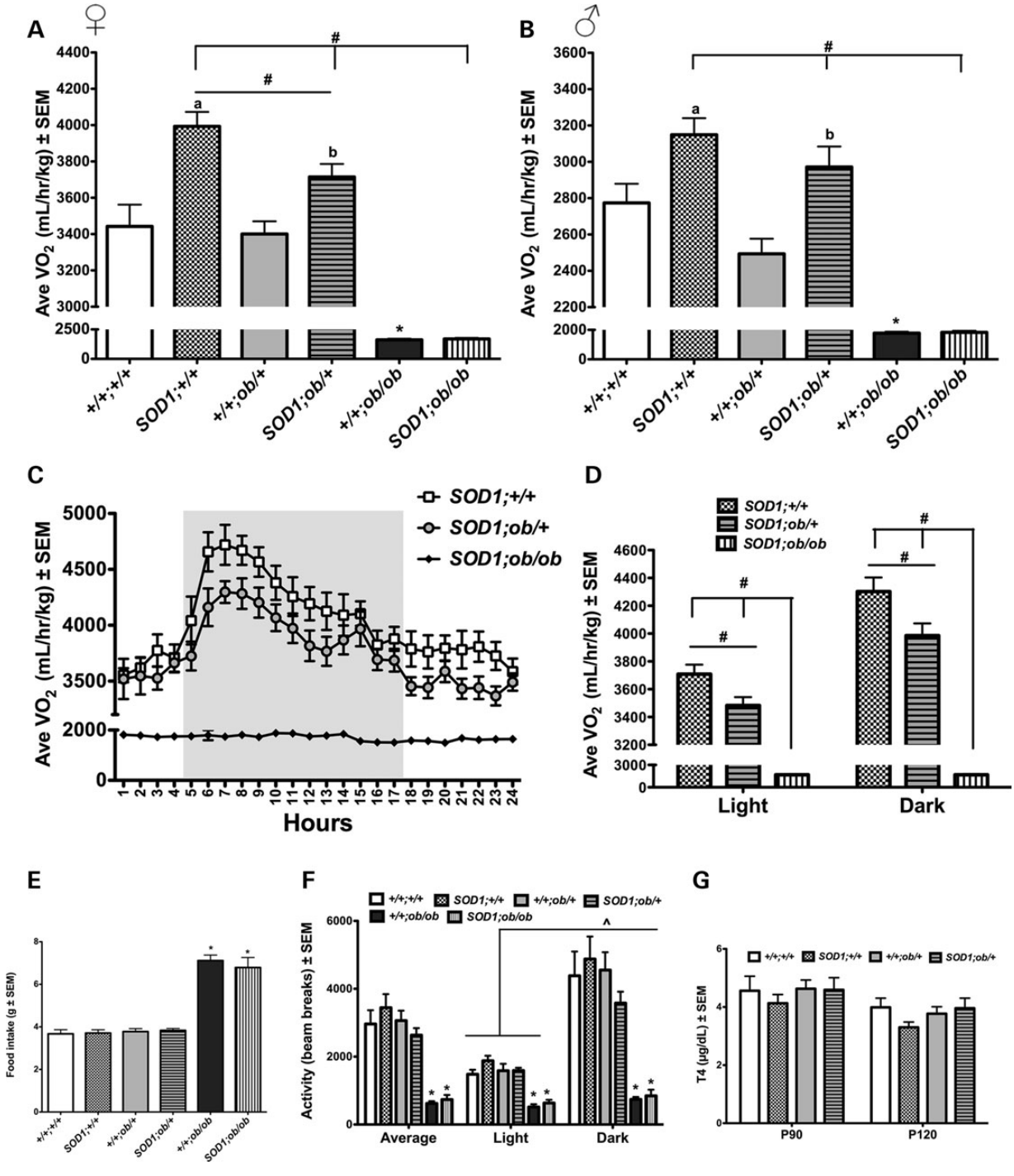

Figure 3. Leptin-deficient, mutant SOD1 females exhibit decreased energy expenditure. Average VO_2 levels in female (**A**) and male (**B**) mice. In both sexes, average VO_2 levels are significantly elevated in *SOD1;+/+* compared with *+/+;+/+* mice ($^{a}P < 0.04$). (A) Although *SOD1;ob/+* females still show elevated energy expenditure compared with *+/+;ob/+* mice ($^{b}P < 0.008$), they demonstrate reduced average VO_2 levels compared with *SOD1;+/+* mice ($^{\#}P = 0.02$). *SOD1;ob/ob* females also show decreased average VO_2 levels compared with *SOD1;+/+* mice ($^{\#}P < 0.0001$), with no difference compared with *+/+;ob/ob* mice. (B) Among males, only *SOD1;ob/ob* mice show reduced VO_2 levels compared with *SOD1;+/+* and *SOD1;ob/+* mice ($^{\#}P < 0.0001$). For both males and females, control *+/+;ob/ob* mice show decreased VO_2 levels compared with all other genotypes ($^{*}P < 0.0001$). For (A) and (B), $n \geq 8$ except for *ob/ob* mice, $n \geq 3$. (**C**) Energy expenditure in P120 mutant SOD1 females across a 24-h time period. Gray shading indicates duration of dark cycle. (**D**) Quantification of data in (C), divided into average VO_2 levels during the light and dark cycles. Reduced VO_2 levels in leptin-deficient mice are found during both cycles ($^{\#}P \leq 0.05$). (**E**) With exemption of leptin null mice ($^{*}P < 0.0001$), all genotypes consumed the same amount of food during a 24-h period. $n \geq 5$ except for *ob/ob* mice, $n \geq 3$. (**F**) With the exemption of leptin null mice ($^{*}P < 0.0001$), there were no significant differences in average activity levels (*X*-axis beam breaks) among genotypes, whether it was during the light or dark cycles. All mice were more active during the dark phase ($\hat{}P \leq 0.0001$). $n \geq 10$ except for *ob/ob* mice, $n \geq 3$. (**G**) There are no differences in serum T4 levels among genotypes. $n \geq 7$. Error bars ± SEM. [a]Significantly different from *+/+;+/+*, [b]significantly different from *+/+;ob/+*, [#]significantly different from *SOD1;+/+* and/or *SOD1;ob/+*, [*]significantly different from *+/+;+/+* and *+/+;ob/+* or *SOD;+/+* and *SOD1;ob/+*, ^ significantly different from light cycle.

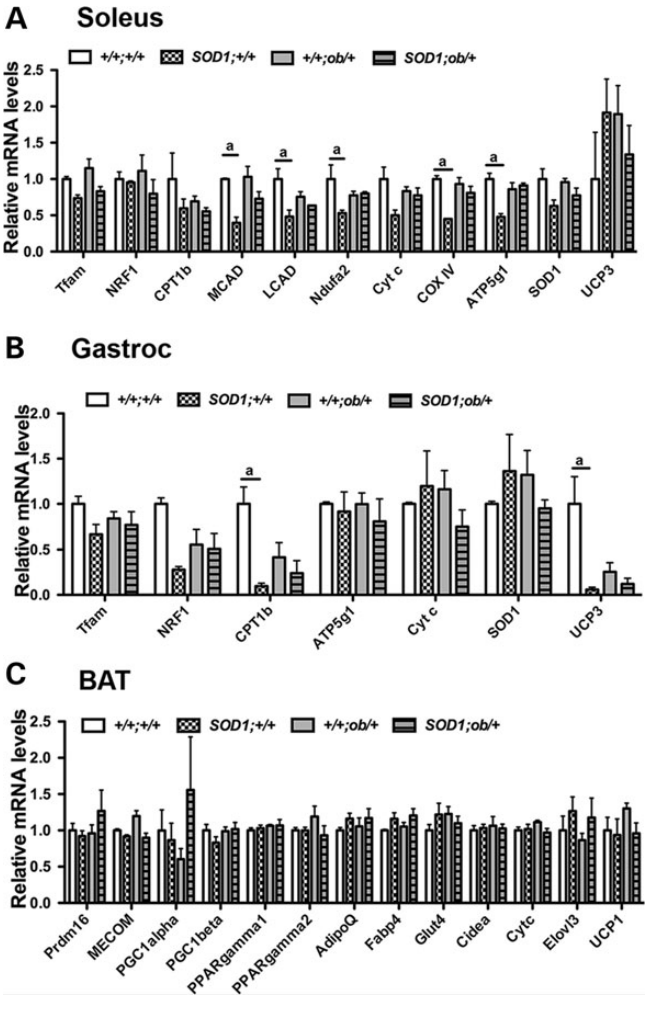

Figure 4. Expression of metabolic genes in skeletal muscle and BAT of mutant SOD1 mice. (**A**) Isolated soleus muscle from P120 mice show significant reductions in transcript expression of genes involved in FAO, such as MCAD and LCAD ($^aP < 0.05$), as well as ETC activity, such as Ndufa2, COXIV and ATP5g1 ($^aP < 0.05$). Partial rescue was obtained in *SOD1;ob/+* mice for MCAD, Ndufa2, COXIV and ATP5g1, although this did not attain significance. (**B**) Isolated gastrocnemius (gastroc) muscle from P120 mice do not show coordinated changes in the transcript expression profiles of key metabolic genes, except significant reductions between +/+;+/+ and *SOD1;*+/+ mice in the expression of CPT1B ($^aP < 0.05$) and UCP3 ($^aP < 0.001$). No rescue was obtained in *SOD1;ob/+* for both genes. (**C**) Isolated BAT do not show changes in gene expression. $n \geq 3$. Error bars ± SEM.

decreased compared with +/+;+/+ mice ($P < 0.01$, and $P < 0.001$, respectively) (Fig. 4B). In neither case did leptin-deficiency modulate these effects. No other changes were detected in the genes surveyed. However, of note, UCP3 levels were also significantly decreased in +/+;*ob/+* compared with +/+;+/+ mice ($P < 0.001$).

We also profiled gene expression in BAT tissue, since BAT physiology can influence bodyweight and energy expenditure (23,24). In all the metabolic genes surveyed, there were no significant changes among genotypes (Fig. 4C). In sum, these results suggest that gene expression changes in skeletal muscle or BAT do not correspond to the hypermetabolic phenotype of mutant SOD1 mice.

Leptin-deficient, mutant SOD1 animals live longer

We next wondered whether the improved organismal metabolism observed in leptin-deficient mice are pathophysiologically relevant to motor neuron disease progression. Does leptin deficiency enhance survival in mutant SOD1 mice? We measured lifespan in mutant mice of both sexes and found that *SOD1;ob/+* female mice live longer than *SOD1;*+/+ counterparts (Fig. 5A; Supplementary Material, Table S3). Compared with *SOD1;*+/+, *SOD1;ob/+* showed increased median (153 versus 170 days in *SOD1;*+/+ and *SOD1;ob/+* mice, respectively, $P < 0.0030$ by Wilcoxon test). Maximum lifespan was also increased (176 versus 193 days in *SOD1;*+/+ and *SOD1;ob/+* mice, respectively). We also monitored disease onset and progression in the mice. Disease onset is defined as the last day of individual peak bodyweight. Early phase of disease is defined as the duration of time between attainment of peak bodyweight and 10% loss of peak bodyweight, while the late phase of disease is defined as the duration of time between loss of 10% of bodyweight until the end stage (25). Disease onset was similar between *SOD1;*+/+ and *SOD1;ob/+* female mice (Fig. 5B; Supplementary Material, Table S3). However, *SOD1;ob/+* mice demonstrated increased duration of both the early ($P = 0.049$), as well as the late phases of disease ($P = 0.0356$) (Fig. 5C). An increase in both the duration of the early and late phases of disease contributed to an overall increase in disease duration of ∼17 days ($P = 0.0226$). Although there was a trend, male *SOD1;ob*,+ mice did not live significantly longer compared with *SOD1;*+/+ counterparts (Fig. 5D; Supplementary Material, Table S3). Disease onset was also similar between *SOD1;*+/+ and *SOD1;ob/+* mice (Fig. 5E). However, male *SOD1;ob/+* mice demonstrated increased duration of the early, but not late, phase of disease ($P = 0.0136$), which contributed towards a modest increase in total disease duration of ∼9 days ($P = 0.0367$) (Fig. 5F). These data suggest that placing mutant SOD1 animals in a leptin-haploinsufficient background slowed the rate of disease progression, and this effect was stronger in females compared with males.

Due to the combination of obesity and hind limb weakness, *SOD1;ob/ob* animals unfortunately developed pressure sores, which necessitated euthanasia prior to limb paralysis (∼146 days). Thus, we were unable to determine whether a complete loss of leptin activity had greater beneficial effects on survival and disease duration than leptin haploinsufficiency.

Leptin-deficient, mutant SOD1 animals demonstrate improved muscle function

We next examined motor function of bigenic (*SOD1;ob/+* and *SOD1;ob/ob*) mice to determine whether leptin deficiency improves the motor phenotype of mutant SOD1 animals. In a test of hind limb grip strength, by ANOVA, there were significant effects of genotype on behavior among female mice across multiple time points: P60 ($F_{(5,55)} = 7.72$, $P < 0.0001$), P90 ($F_{(5,45)} = 12.06$, $P < 0.0001$) and P120 ($F_{(5,33)} = 54.52$, $P < 0.0001$). Female *SOD1;*+/+ mice showed progressive worsening of hind limb grip strength, and this was less severe in *SOD1;ob/+* mice (Fig. 6A). *SOD;ob/+* animals showed a 20% improvement at P90 ($P = 0.03$), which increased to >40% at P120 ($P < 0.0001$). *SOD1;ob/ob* mice demonstrated

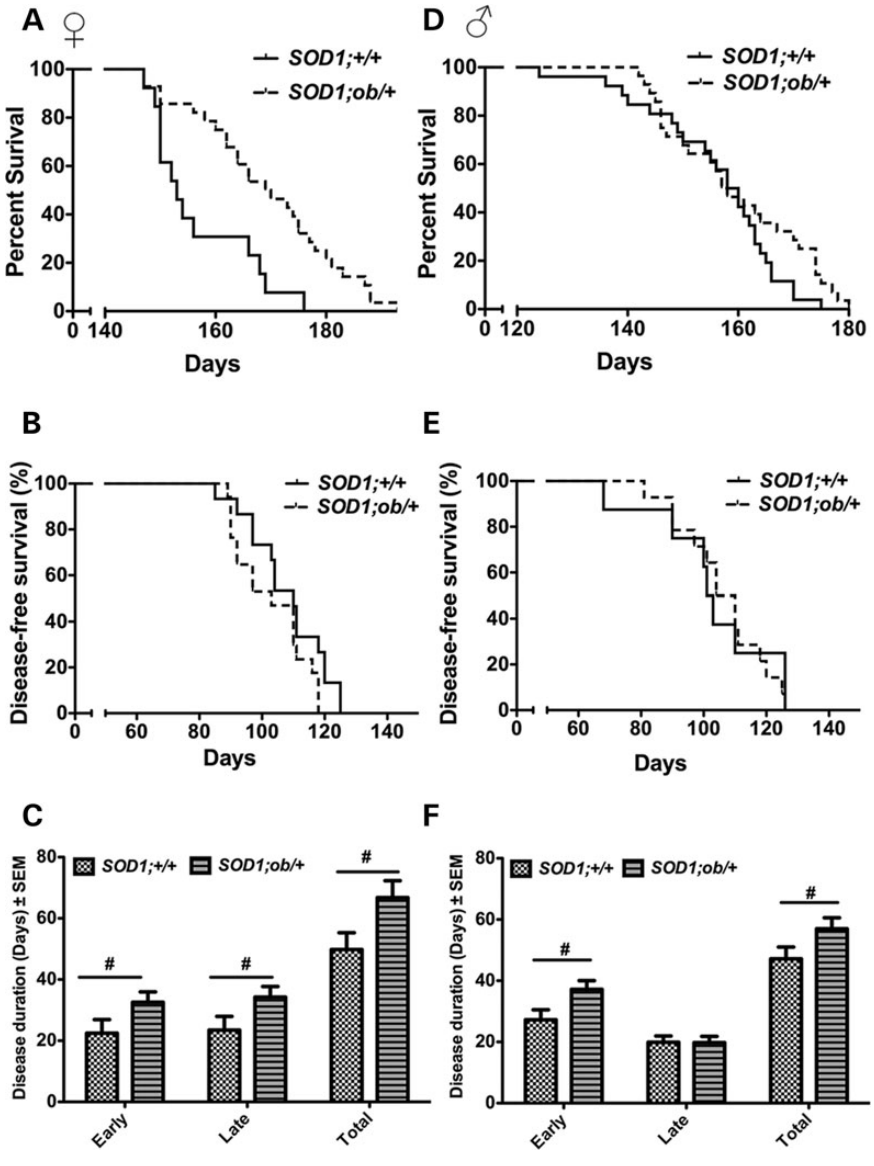

Figure 5. Leptin-deficient, mutant SOD1 mice demonstrate increased survival. (**A**). Kaplan–Meir survival plots for female mice. *SOD1;ob/+* females live longer than *SOD1,+/+* mice ($P = 0.0030$ by Wilcoxon test). $n \geq 13$. (**B**) Kaplan–Meir survival plots for disease onset in female mice. Between genotypes, there is no significant difference in disease onset, as measured by the day that peak bodyweight was attained ($P = 0.7035$ by Wilcoxon test). (**C**) Compared with *SOD1;+/+*, *SOD1;ob/+* females show increased duration of the early ($^{\#}P = 0.0409$) and late phases of disease ($^{\#}P = 0.0356$), as well as total disease duration ($^{\#}P = 0.0226$). $n \geq 8$. (**D**) Kaplan–Meir survival plots for male mice. *SOD1;ob/+* males do not live significantly longer than *SOD1;+/+* mice ($P = 0.4202$ by Wilcoxon test). $n \geq 26$. (**E**) Kaplan–Meir survival plots for disease onset in male mice. There is no significant difference in disease onset between male genotypes ($P = 0.1622$ by Wilcoxon test). (**F**) Compared with *SOD1;+/+*, *SOD1;ob/+* males show increased duration of the early ($^{\#}P = 0.0136$) but not late phase of disease ($P = 0.4868$), as well as an overall increase in disease duration ($^{\#}P = 0.0367$). $n \geq 15$. Error bars ± SEM.

an even larger improvement in grip strength compared with *SOD1;+/+*, as early as P60 ($P < 0.01$), which continued to P120 ($P < 0.0001$). Similar results were observed for males (Fig. 6C). The *+/+;+/+* and *+/+;ob/+* mice had equivalent grip strength at all-time points for either sex (Fig. 6A and C).

Mice were also subjected to a treadmill test to assess muscle endurance. There were significant effects of genotype on behavior among female mice at P60 ($F_{(3,39)} = 3.91$, $P < 0.02$), P90 ($F_{(3,32)} = 7.92$, $P < 0.0004$) and P120 ($F_{(3,19)} = 11.78$, $P < 0.0001$). *SOD1;+/+* mice performed worse than *+/+;+/+* mice at P90 and P120 ($P < 0.005$ and $P < 0.0001$, respectively) (Fig. 6B). However, *SOD1;ob/+* ran significantly longer than *SOD1;+/+* mice (65.3 ± 25.6 min versus 9.8 ± 3.06 min, respectively, $P < 0.02$) at P120, but not at P60 and P90. Similar impairments were observed in mutant SOD1 males, although no significant improvements were found in *SOD1;ob/+* males (Fig. 6D). The *+/+;ob/ob* and *SOD1;ob/ob* animals were too obese to perform this assay and were therefore excluded. In non-Tg mice, no significant differences in treadmill performance were noted. At a later time point of disease (~P150), we also observed home cage and tail suspension behaviors in mutant mice and found that *SOD1;ob/+* mice were more

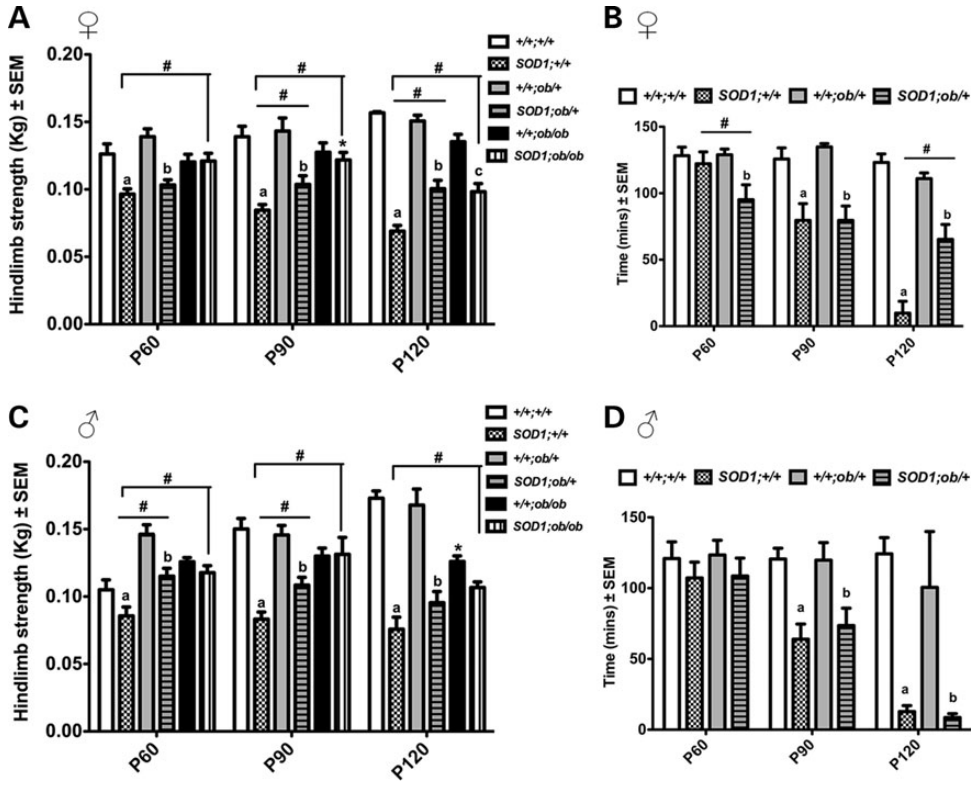

Figure 6. Leptin-deficient, mutant SOD1 mice display improved motor function. (**A**) Mutant SOD1 females show progressive hind limb weakening compared with non-Tg controls ($^{a}P < 0.0001$, $^{b}P < 0.002$ and $^{c}P < 0.0003$). Compared with *SOD1;+/+* mice, *SOD1;ob/+* and *SOD1;ob/ob* females possess improved hind limb grip strength at several disease time points ($^{\#}P < 0.01$). Except for a modest decrease in hind limb grip strength of *+/+;ob/ob* compared with *+/+;ob/+* animals at P90 ($^{*}P = 0.02$), there are no other differences among non-Tg controls. (**B**) Compared with non-Tg mice, both female *SOD1;+/+* and *SOD1;ob/+* mice show decreased time on the treadmill ($^{a}P < 0.005$, $^{b}P < 0.03$); however, P120 *SOD1;ob/+* mice run significantly longer than *SOD1;+/+* mice ($^{\#}P < 0.02$). $n \geq 10$. (**C**) Mutant SOD1 males show progressive hind limb weakening compared with non-Tg controls ($^{a}P < 0.0001$, $^{b}P < 0.006$). Compared with *SOD1;+/+* mice, *SOD1;ob/+* and *SOD1;ob/ob* males possess improved hind limb grip strength at several disease time points ($^{\#}P \leq 0.05$). P120 *+/+;ob/ob* males show decreased hind limb strength compared with other non-Tg controls ($^{*}P \leq 0.003$). For all hind limb tests, $n \geq 10$, except in *+/+;ob/ob* and *SOD1;ob/ob* animals, $n = 4$. (**D**) *SOD1;+/+* and *SOD1;ob/+* males show decreased time on the treadmill compared with non-Tg controls ($^{a}P \leq 0.0005$, $^{b}P \leq 0.03$). $n \geq 4$. Error bars ± SEM. ^{a}Significantly different from *+/+;/+/+*, ^{b}significantly different from *+/+;ob/+*, ^{c}significantly different from *+/+;ob/ob*, $^{\#}$significantly different from *SOD1;ob/+* and/or *SOD1;ob/ob*, * significantly different from *+/+;+/+* and *+/+;ob/+* or *SOD;+/+* and *SOD1;ob/+*.

ambulatory and active compared with *SOD1;+/+* mice (Supplementary Material, Movies S1 and S2). These data indicate that leptin-deficient, mutant SOD1 mice possess improved motor strength and endurance, and these improvements occur both at early and later stages of disease.

Motor neuron loss is blunted in leptin-deficient, mutant SOD1 animals

Next, we investigated whether the beneficial effects of placing mutant SOD1 animals in a leptin-deficient background is also neuroprotective. To address this question, we counted motor neurons in the lumbar spinal cord, the spinal cord segment which shows the earliest signs of motor neuron degeneration. At P90, there were no significant differences among genotypes; however, at P120, there was a significant effect of genotype on motor neuron number ($F_{(3,11)} = 6.16$, $P = 0.01$) (Fig. 7A). *SOD1;+/+* displayed a 10% loss of lumbar spinal cord motor neurons compared with *+/+;+/+* controls (258 ± 3.0 versus 278 ± 6.8 motor neurons, respectively, $P < 0.007$), and this was completely rescued in *SOD1;ob/+* mice ($P = 0.003$). These data indicate that placing mutant SOD1 mice in a leptin-deficient background protects against motor neuron death.

Localization of the leptin receptor in the ventral horn of the spinal cord

Since reduction in circulating leptin levels showed benefits in the mutant SOD1 model and leptin has central nervous system actions (26,27), we wondered about the distribution of leptin receptors in the spinal cord. Using an antibody against the best characterized signal transducing leptin receptor (Ob-Rb), we immunostained spinal cord sections from wild-type, non-Tg mice. Ob-Rb immunoreactivity was seen in a minority of cells in the ventral horn that were clearly not alpha motor neurons (Fig. 7B). The immunopositive cells had small cell bodies with processes extending into the neuropil near motor neurons. Based on their morphology, Ob-Rb immunoreactive cells appear to be glial, either a subpopulation of oligodendrocytes or microglia. These observations suggest that any change in leptin signaling in the spinal cord in the mice we have studied is unlikely to be due to a direct effect on alpha motor neurons.

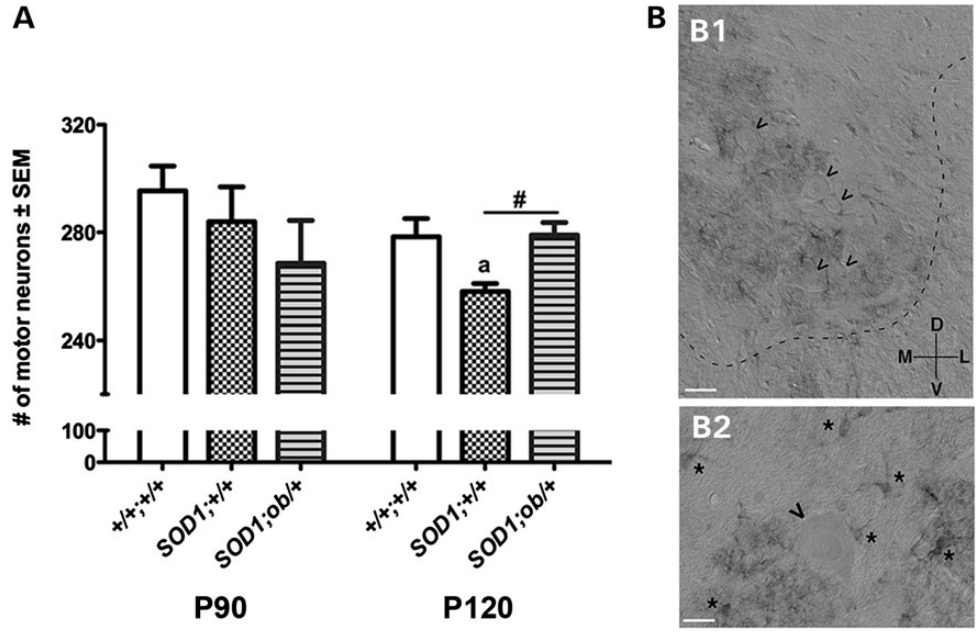

Figure 7. Leptin-deficient, mutant SOD1 mice display rescued motor neuron loss despite lacking expression of the leptin receptor Ob-Rb by motor neurons. (**A**) At P120, but not P90, significant motor neuron loss occurs in the lumbar spinal cord of *SOD1;+/+* compared with *+/+;+/+* mice ($^{a}P < 0.007$). Motor neuron loss is rescued in *SOD1;ob/+* mice ($^{\#}P = 0.003$). $n \geq 3$. Error bars ± SEM. (**B**) Ob-Rb localization in spinal ventral horn. (B1) Immunohistological staining of the spinal cord for the leptin receptor Ob-Rb. The ventral horn is shown and the gray–white junction is outlined with a dashed line. The orientation is shown in the lower left: 'D', dorsal; 'V', ventral; 'M', medial and 'L', lateral. In this differential contrast image, motor neurons are highlighted by the '<' symbol and at least 5 are visible in this focal plane. Numerous small immunoreactive cells are seen but motor neurons are not immunostained. Calibration bar = 45 μm. (B2) Higher power image of a single motor neuron (identified by '<' symbol) and many small immunoreactive cells (identified by '*' symbol). These small immunopositive cells bear processes and several are juxtaposed to the motor neuron. Calibration bar = 23 μm.

DISCUSSION

Metabolic abnormalities have been reported in ALS patients and mouse models (4,13). Here, we present evidence that motor neuron disease can be mitigated by reducing circulating leptin levels, which in turn, alter organismal metabolism. First, we confirm the presence of whole-animal metabolic defects in G93A mutant SOD1 mice, a well-characterized model of fALS (20). Second, we demonstrate that a genetic manipulation, which improves organismal energy homeostasis, is both functionally beneficial and neuroprotective. Placing mutant SOD1 mice in a leptin-deficient (*ob/ob* and *ob/+*) background, a mouse model of hypometabolism, decreased the rate of weight loss, increased WAT stores, and reduced energy expenditure. Moreover, these beneficial effects coincided with enhanced survival, improved motor behavior, and decreased motor neuron degeneration. Third, changes in organismal metabolism were not associated with alterations in food intake, activity levels, BAT weight and serum thyroid hormone levels. Fourth, we report sex differences, especially in energy expenditure and survivorship, which have not been considered in previous metabolic studies (6,13). Whether the observed beneficial effects are mediated by leptin-deficiency *per se* or a combination of alterations in organismal metabolism and leptin deficiency is not immediately evident. Further studies are needed in order to clearly dissect these two possible mechanisms. Since SOD1-causing mutations represent only a minor subset of ALS patients, future experiments in SOD1-independent mouse models will also be important.

This study provides the first genetic evidence that manipulating organismal metabolism modifies motor neuron disease progression. Epidemiological studies have shown that increased fat mass and dyslipidemia are associated with slower disease progression in ALS patients (9,28,29). Furthermore, a retrospective study reported that the presence of premorbid diabetes mellitus type 2 in ALS patients was associated with delayed disease onset (30). It is interesting that this 'beneficial' metabolic profile is reminiscent of the phenotypes of leptin-deficient *ob/ob* animals, and to a certain extent, *ob/+* animals (16,19,31). It is worth noting that leptin antagonists have been clinically used to treat cachexia in a mouse model of chronic kidney disease (32).

Our results confirm and extend previous reports showing mutant SOD1 mice benefit from a high-calorie diet (6,13). The terminology 'high-calorie diet' is broadly used, and in fact, the dietary content and beneficial effects seen in these studies varied significantly. For example, compared with a standard rodent diet (22% fat, 55% carbohydrates and 23% protein), a high-calorie diet in one study that reported increased survival in mutant SOD1 mice consisted of 20% butter fat and 0.15% cholesterol (13). In another study that reported improved muscle function, but no change in survival, a high-calorie diet consisted of 60% fat, 20% carbohydrates and 20% protein (6). These observations raise the possibility that the provision of specific nutrient(s) enriched in these diets, not increased calories *per se*, contribute to the observed beneficial effects. Prior studies, independent of mutant SOD expression, have emphasized that different 'high-calorie diets' vary in their effects on organismal metabolism, and this specifically depends on the composition

of the diet rather than the total calorie intake (33–35). An advantage of our experimental design is that all groups consumed the same standard rodent diet. It is noteworthy that *SOD1;+/+* and *SOD1;ob/+* mice consumed equal amounts of food (3.7 g/day). Our study demonstrates that by reducing energy expenditure (by reducing leptin levels), the distorted organismal level energy economy in the mutant SOD1 mice is corrected, and this is beneficial in this model of ALS.

In this report, we also observed sex differences of our genetic manipulation. Leptin-deficient *SOD1;ob/+* females show increased survival, improved motor function and decreased energy expenditure in comparison to *SOD1;+/+* animals. In contrast, leptin-deficient *SOD1;ob/+* males display a more modest increase in survival and reduction in energy expenditure. Furthermore, only *SOD1;+/+* males, not *SOD1;+/+* females, showed decreased serum leptin levels compared with non-Tg counterparts. Typically, WAT depot size correlates with circulating leptin levels (36). However, leptin levels are influenced by a number of factors, including various hormones (i.e. glucocorticoids, sex and reproductive hormones) (37), several of which are altered in mutant SOD1 mice (13). It is likely that these hormonal changes may mask alterations in circulating leptin levels as a function of decreased WAT mass. These aforementioned results are intriguing for a number of reasons. First, these results confirm previous observations of sex-dependent differences in ALS disease progression, in which the underlying mechanisms remain enigmatic (38–41). Second, these results suggest an interaction between sex, metabolic dysfunction and motor neuron disease progression. Sexual dimorphic responses to various experimental manipulations have previously been reported in ALS patients and mouse models (41–44). Ovariectomized female G93A mutant SOD1 mice demonstrate worsened disease progression; moreover, disease symptoms are attenuated by estradiol treatment (45,46). Since estrogen plays an important role in mitochondrial function and aging (47), these observations may indicate that sex-dependent manipulations may be influenced by the interaction between estrogen and mitochondrial metabolism. Along the same lines, a recent report describes sex-specific effects of the transcriptional co-activator and metabolic regulator peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) on mutant SOD1-induced disease progression and links this observation to differential expression of the neurotrophic factor VEGF and estrogen receptor ERRα in the nervous system (41). It will be interesting to further investigate how leptin deficiency affects mitochondrial function in both sexes as well as how PGC1α and its downstream targets are affected in leptin-deficient mutant SOD1 animals.

One major question raised by this work is whether further amelioration of mutant SOD1-induced motor neuron disease could be achieved with a greater or lesser reduction in serum leptin levels. One of the limitations of this genetic experiment is the inability to precisely regulate leptin levels. In our bigenic mice, leptin has been reduced from birth to either ∼50% or 0% of wild-type levels. We show that in the context of motor neuron disease, even a 50% global decrease in serum leptin levels is associated with improvements in both metabolic and neuromuscular functions. Observations in the *SOD1;ob/ob* mice hint at the possibility that further reductions in serum leptin levels may have a more pronounced beneficial effect. These observations are consistent with recent work showing that blocking spinal cord leptin activity can reverse and prevent pain behaviors induced by sciatic nerve injury (48). The long form of the leptin receptor (Ob-Rb), which is thought to mediate majority of leptin's biological signals (37,49), has been reported to be expressed in the spinal cord, although localized to either sensory neurons or astrocytes (48). Our immunohistological staining result does not suggest Ob-Rb expression by motor neurons and instead suggests possible expression by glial cells. Leptin signaling may indirectly affect motor neuron survival by modulating signaling pathways in astrocytes, which in turn has been shown to play a role in motor neuron disease progression (50). However, since leptin can be neurotrophic (at least during development) (51), reducing leptin signaling may have unintended consequences. Manipulations that more tightly regulate leptin levels should be considered for the future.

Our study begins to shed light on which tissues may be responsible for improved energy homeostasis. Contrary to previous reports (21,22), we do not find evidence that changes in gene expression in skeletal muscle correspond with the hypermetabolic phenotype of mutant SOD1 mice. We found that FAO and ETC gene expression were significantly reduced, particularly in soleus muscle, of mutant SOD1 mice at a time point when hypermetabolism is present. This result is consistent with previous observations of impaired muscle mitochondrial respiration (22). We also found that UCP3 expression was reduced, suggesting that uncoupled energy in the form of heat, may not be contributing to elevating energy expenditure. The disparity between our results and previous reports may have several sources. Our mice were congenic (C57BL/6), whereas previous reports utilized mixed FVB/N or SJL-B6 strains (13,22,52). Background genotype is well known to influence metabolic parameters (53,54), as well as disease progression in mutant SOD1 mice (55,56). In addition, at the time point tested, muscle denervation has already occurred, which may confound interpretation of the observed metabolic gene profile (57). Given our observations that mutant SOD1 mice are hypermetabolic at this time point, it is possible that other tissue(s) may significantly contribute to elevating energy expenditure. Further investigation is required to see if tissues, including liver, brain and even bone, may influence whole-animal metabolism in this mutant SOD1 mouse model of ALS.

Interesting recent work attempted to manipulate energetic status of muscle in mutant SOD1 mice by transgenic overexpression of PGC1α, a master regulator of mitochondrial biogenesis (58). This manipulation rescued some motor defects, but did not increase survival. In another experiment, Eschbach and colleagues placed mutant SOD1 mice in the background of PGC1α-deficient mice and found decreased survival in the bigenic mice (41). The effects of these manipulations on organismal metabolism remain to be explored. We favor the view that impairment in muscle mitochondrial function may be a significant contributor to motor weakness in the mutant SOD1 model of ALS, but its concrete role in contributing to organismal dysfunction in this model requires further query.

In sum, our results support the hypothesis that organism-level metabolic dysfunction is pathophysiologically relevant to motor neuron disease. Manipulating leptin activity and other metabolic pathways that ultimately alter body composition and energy expenditure may be intriguing therapeutic targets to treat ALS patients.

MATERIALS AND METHODS

Mouse care

Since female G93A mutant SOD1 mice are poor breeders, male hemizygous G93A mutant SOD1 mice on the C57BL/6 background (Strain # 004435, Jackson Laboratory, Bar Harbor, ME, USA) were crossed with female mice heterozygote for the obese gene (*ob*/+) (Strain # 000632, Jackson Laboratory). From the F1 generation, mutant *SOD1;ob*/+ males were crossed with *ob*/+ females to generate the F2 generation. The genotypes of mutant SOD1 mice were determined by PCR using tail snip DNA, as described elsewhere (59), while the genotypes of *ob* mice were determined by PCR using tail snip DNA and a subsequent restriction enzyme digest as described on the Jackson Laboratory website. Mice were fed a standard diet (23% protein, 22% fat, 55% carbohydrates) and were housed at 22°C on a 12-h light–dark cycle. All animals were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Analyses of disease onset and survival

The early and late stages of disease were determined as previously described (25,60). Disease onset is defined as the last day of individual peak bodyweight before gradual loss occurs. The stages of disease are defined as follows: the early stage of disease is defined as the duration of time between peak bodyweight until loss of 10% of peak bodyweight. The late stage of disease is defined as the duration of time between 10% loss of peak bodyweight until the end stage of disease. The end stage of disease is defined as the day when an animal could no longer right itself within 30 s for three consecutive trials when placed on its side. Animals were euthanized at the end stage of disease.

Motor behavior

All tests were performed at P60, P90 and P120. Mice were habituated to the procedure room before testing. Grip strength assays were performed as previously described (60). Endurance was tested using a mouse treadmill apparatus (Columbus Instruments). Mice were tested on the following speed categories for a total time of 140 min: 80 mm/s (20 min), 120 mm/s (60 min) and 170 mm/s (60 min). The endpoint of testing was defined as when an animal ceased to run, even when gently prodded with a wooden tongue depressor. The total time duration on the treadmill (sum of all three speed categories) was analyzed for each mouse.

Body composition measurements

Bodyweights were assessed weekly for at least 13 weeks. BAT and gonadal WAT were dissected and weighed at the indicated age. Total lean mass, % of WAT and BMD were determined by DEXA (PIXImus DEXA; GE). DEXA imaging was performed by the Mouse Phenotying, Physiology and Metabolism Core at the University of Pennsylvania.

Indirect calorimetry, food intake and activity

Animals were initially weighed and acclimated to the test cage. Volume oxygen (VO_2) and volume carbon dioxide production (VCO_2) were measured every ~20 min using the Oxymax Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments) and are reported as average VO_2 per hour normalized to bodyweight (mL/h/kg). Using the CLAMS machine, activity counts by infrared beam interruptions and food intake were simultaneously measured. More specifically, food intake was measured by deducting the weight of powderized food pellets at the end of experimentation from the starting weight at the beginning of experimentation. To complement this experiment and to control for a novel environment that may affect feeding behavior, we also performed a more 'manual' experiment, wherein a set weight of food pellets was placed at the same time each day into a clean home cage, which held a mouse. The next day the weight of the remaining pellets was recorded and deducted from the starting weight. This experiment was performed for 14 days straight. The bodyweight of each mouse was also recorded daily. Results for each genotype were similar to that acquired from the CLAMS.

Leptin and thyroid hormone 4 serum measurements

Retro-orbital bleeds were performed from *ad libitum* fed P90 and P120 mice using microhematocrit capillary tubes (Fisherbrand). Blood was allowed to coagulate for 15 min at room temperature, and spun at $7500 \times g$ for another 15 min. Serum was collected and stored in −80°C until use. Leptin and thyroid hormone 4 (T4) assays were performed by the University of Pennsylvania Radioimmunoassay/Biomarkers Core Facilities, as described elsewhere using a coated tube assay for T4 measurements (MPBiomedicals) and a sandwich ELISA for leptin measurements (EMD Millipore).

Quantitative real-time PCR

Muscle was homogenized using the Qiagen Tissue Lyser II and RNA was isolated using the TRIzol method (Invitrogen). Adipose tissue was homogenized on ice using an electric homogenizer, and RNA was isolated using the TRIzol method combined with Qiagen RNEasy mini columns. Total RNA was reverse transcribed using the ABI high-capacity cDNA synthesis kit (Applied Biosystems) or Qiagen Quantiscript Kit. Real-time PCR analysis was performed as previously described for muscle (61) and for adipose tissue (62). All primers are available upon request.

Motor neuron counts

Mice were sacrificed at P90 and P120. After perfusion fixation with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4), the spinal cord from lumbar segment 1 to sacral segment 1 (L1–S1) was dissected out of the vertebral canal, post-fixed overnight at 4°C, and then cryoprotected by overnight immersion in 30% sucrose in 0.1 M phosphate buffer (pH = 7.4). Using the Jung-Frigocut 2800N cryostat (Leica), consecutive 40 μm-thick sections were collected and Nissl stained. Motor neurons were identified by their location in

Rexed lamina IX and characteristic size and polygonal morphology. We counted the number of motor neurons in every 6th spinal cord section from an individual animal and these values were summed. In previous work, we found that this estimate of the number of motor neurons can be reliably used to make group comparisons (63).

Immunohistology

P90 C57Bl/6 mice were perfused and fixed according to the protocol described above. The following day post-fixation, the tissue was blocked into 2–3 mm segments, frozen in dry ice and cryostat sections (10 μm) were prepared with a Jung Frigocut 2800N (Leica). Tissue sections were thaw mounted on Superfrost® Plus slides (VWR), permeabilized in PBS + 1% Triton X-100 (for 10 min), blocked in PBS + 5% fetal calf serum, FBS (for 1 h) and extensively washed with PBS. Slides were incubated with a rabbit antibody directed against mouse leptin receptor Ob-Rb (Alpha Diagnostics, catalogue # OBR12-A) at 1:1000 in PBS + 5% FBS overnight at room temperature in a sealed humidified chamber. Control slides were incubated with PBS + 5% FBS. The following day, the slides were washed in PBS, incubated with biotinylated anti-rabbit antibody (Amersham Pharmacia Biotech) diluted 1:250 in PBS + 5% FBS for 3 h. This was followed by PBS washing, incubation with ABC reagent (Vector), and visualized with 0.03% 3,3′-diaminobenzidine HCl and 0.003% H_2O_2. Slides were dehydrated, defatted with xylene, coverslipped with Permount and imaged on an Axio Imager.A2 (Zeiss).

Statistics

Statistics were analyzed using GraphPad Prism 5.0. Student's *t*-tests were used in comparisons between two-group data and multivariate ANOVAs, including repeated measures ANOVAs, were used in comparisons among three or more groups of data. *Post hoc* tests were performed to confirm significance. Nonparametric statistical analysis, including Kruskal–Wallis tests, was used to analyze behavior from the treadmill tests. Kaplan–Meier survival plots and Gehan–Breslow–Wilcoxon tests were utilized to analyze lifespan. The threshold for significance was set at $P \leq 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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