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Hdac6 deletion delays disease progression in the *SOD1^{G93A}* mouse model of ALS

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Defects in axonal transport are thought to contribute to the pathogenesis of neurodegenerative disease. Because α-tubulin acetylation facilitates axonal transport, inhibition of the α-tubulin deacetylating enzymes, histone deacetylase 6 (Hdac6) and silent information regulator 2 (Sirt2), is thought to be an interesting therapeutic strategy for these conditions. Amyotrophic lateral sclerosis (ALS) is a one such rapidly progressive and fatal neurodegenerative disorder, in which axonal transport defects have been found *in vitro* and *in vivo*. To establish whether the inhibition of Hdac6 or Sirt2 may be of interest for ALS treatment, we investigated whether deleting *Hdac6* or *Sirt2* from the superoxide dismutase 1, *SOD1^{G93A}* mouse affects the motor neuron degeneration in this ALS model. Deletion of *Hdac6* significantly extended the survival of *SOD1^{G93A}* mice without affecting disease onset, and maintained motor axon integrity. This protective effect was associated with increased α-tubulin acetylation. Deletion of *Sirt2* failed to affect the disease course, but also did not modify α-tubulin acetylation. These findings show that Hdac6, rather than Sirt2, is a therapeutic target for the treatment of ALS. Moreover, Sirt2 appears not to be a major α-tubulin deacetylase in the nervous system.

INTRODUCTION

Axonal transport defects are thought to contribute to the pathogenesis of neurodegenerative disorders and restoring transport is suggested to be a therapeutic strategy. Post-translational modification of microtubules is a major regulator of axonal transport. One such modification, acetylation of α-tubulin, has been studied intensively in neurodegeneration. It promotes axonal transport by recruiting the molecular motor proteins kinesin-1 and cytoplasmic dynein to microtubules (1,2). Decreased acetylation of α-tubulin underlies the transport defects in Huntington's disease (HD) and Charcot–Marie–Tooth disease (CMT) (1,3). Moreover, neuroprotective effects of genetic or pharmacological inhibition of Hdac6 and Sirt2, the two major α-tubulin deacetylases, have been reported in models of HD, CMT and Parkinson's disease (PD) (1,3–6). However, these enzymes have several other functions in the cell. For instance, Sirt2 has recently been suggested to play a role in programmed necrosis (7). In particular however, Hdac6 plays a major role in the degradation of misfolded proteins that play a causal role in the mechanism of neurodegeneration. It can bind ubiquitinated proteins and dynactin simultaneously (8). It thus enhances transport of ubiquitinated proteins by the cytoplasmic dynein motor to the microtubule-organizing center where these are degraded through the autophagy machinery (9). This ubiquitin-dependent function of Hdac6 is dependent upon the α-tubulin deacetylase activity of this enzyme (8,9). Thus, deacetylase inhibition may have deleterious effects as well. It is therefore necessary to evaluate the net effect of the inhibition of these multifunctional enzymes before accepting them as favorable therapeutic targets.

Amyotrophic lateral sclerosis (ALS) is a progressive, incurable and fatal condition characterized by the loss of upper and lower motor neurons. Its molecular pathogenesis is multifactorial and modified by non-neuronal cells (10,11). In animal models of ALS, axonal transport defects are present early in life, well before the onset of clinical deficits (12,13). The relevance of axonal transport dysfunction for human ALS is

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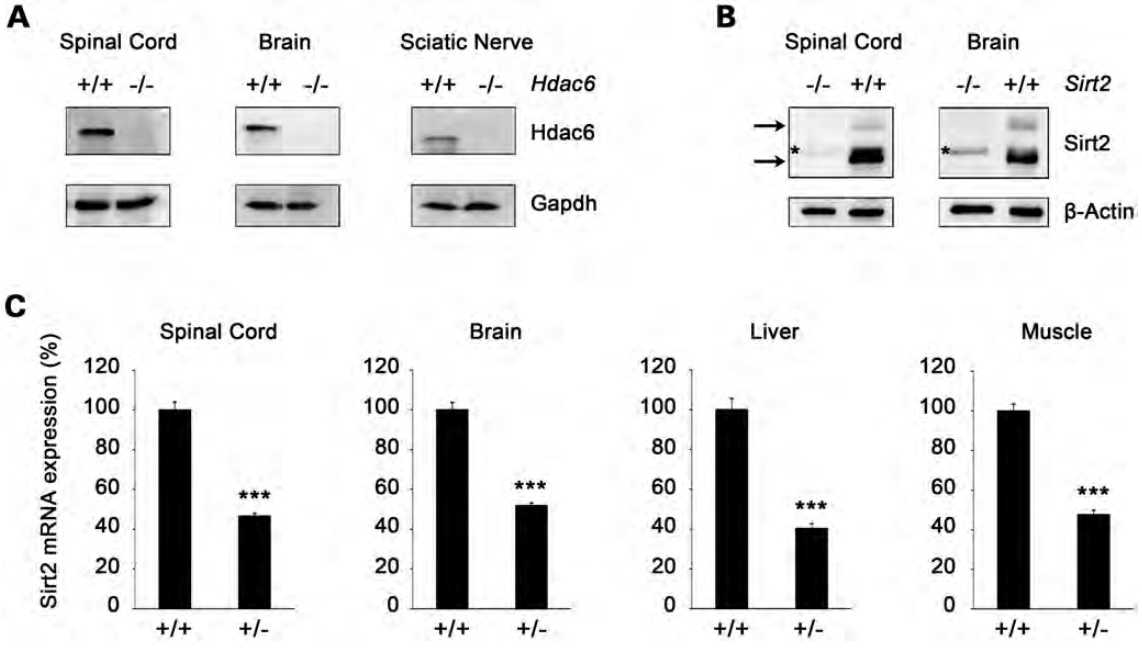

Figure 1. Expression of Sirt2 and Hdac6 in nervous tissue of KO mice. (**A**) Hdac6 protein expression in 90-day-old *Hdac6* KO mice. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a loading control. (**B**) Sirt2 protein expression in 180-day-old *Sirt2* KO mice. Arrows indicate 39 kDa and 43 kDa isoforms of Sirt2 and asterisks indicate a non-specific band. β-Actin was used as a loading control. (**C**) Different panels show Sirt2 mRNA expression levels in various tissues of 80-day-old *Sirt2* KO mice. Values are normalized to the average of five mRNA controls [DNA-directed RNA polymerase II A (Polr2a), hypoxanthine phosphoribosyltransferase 1(Hprt1), ribosomal protein, large, P0 (Rplp0), Gapdh and β-actin] and represented as the percentage of mean ± SEM ($n = 5–6$). The signal for Sirt2 mRNA in $Sirt2^{-/-}$ mice was below detection limits. Student's *t*-test, $^{***}P < 1 \times 10^{-5}$.

demonstrated by the occurrence of spheroids, axonal swellings packed with filamentous material, in familial and sporadic ALS (14,15). In order to elucidate whether deacetylase inhibitors may be a promising avenue for intervention in ALS, we investigated the effect of *Hdac6* and *Sirt2* deletion on the motor neuron degeneration in the superoxide dismutase 1, $SOD1^{G93A}$, mouse model of ALS.

RESULTS

Hdac6 or *Sirt2* knockout does not affect neuromuscular function in mice

In this study, we have used *Sirt2* knockout (KO) mice, obtained by mating conditional *Sirt2* KO mice (16) with CMV-Cre mice, and *Hdac6* KO mice (17). We confirmed the absence of Hdac6 and Sirt2 protein from nervous tissues of *Hdac6* KO and *Sirt2* KO mice, respectively (Fig. 1A and B). In wild-type mice, mainly the smaller 39 kDa isoform of Sirt2 was expressed as reported before (18). Analysis of Sirt2 mRNA expression levels revealed a complete loss of Sirt2 in homozygous *Sirt2* KO mice ($Sirt2^{-/-}$) and a ~50% decrease in heterozygous *Sirt2* KO mice ($Sirt2^{+/-}$) compared with control littermates ($Sirt2^{+/+}$) (Fig. 1C). As a result of the location of the *Hdac6* gene on the X-chromosome, there is variable protein expression in heterozygous females as a result of lionization, and male mice—who share only one X-chromosome—are either non-transgenic or full KO. Therefore, no heterozygous *Hdac6* KO mice were used in this study.

We first excluded that the deletion of either *Hdac6* or *Sirt2* results in a neuromuscular phenotype on itself. Motor performance was evaluated using an accelerating rotarod and was not different between *Hdac6* KO mice and control mice (Supplementary Material, Fig. S1A) or between *Sirt2* KO and control mice (Supplementary Material, Fig. S1B). The compound muscle action potential (CMAP), an electrophysiological measure of neuromuscular innervation, of *Hdac6* KO and *Sirt2* KO mice was not different from control mice (Supplementary Material, Fig. S1C and D). To exclude more subtle changes, we quantified the number of neurons in the ventral horn of lumbar spinal cord as well as the fraction of innervated neuromuscular junctions (NMJs) in the gastrocnemius muscle. No differences in these parameters were detected (Supplementary Material, Fig. S2).

Effect of deletion of *Hdac6* on the course of motor neuron degeneration in $SOD1^{G93A}$ mice

Hdac6 KO mice were crossbred with $SOD1^{G93A}$ mice. As disease onset and survival of $SOD1^{G93A}$ mice vary between litters and genders (19) and female $Hdac6^{-/-}$ mice do not have female control littermates within the same litter, only male mice were included in this study. Age at disease onset, defined by failure on the paw grip endurance (PaGE) test, was not affected by the absence of Hdac6: it was 132 ± 2 days for $SOD1^{G93A}/Hdac6^{Y/+}$ ($n = 25$) and 135 ± 2 days for $SOD1^{G93A}/Hdac6^{Y/-}$ ($n = 26$) mice ($P = 0.236$) (Fig. 2A). In contrast, deletion of *Hdac6* significantly prolonged survival of $SOD1^{G93A}$ mice from 150 ± 2 days for $SOD1^{G93A}/$

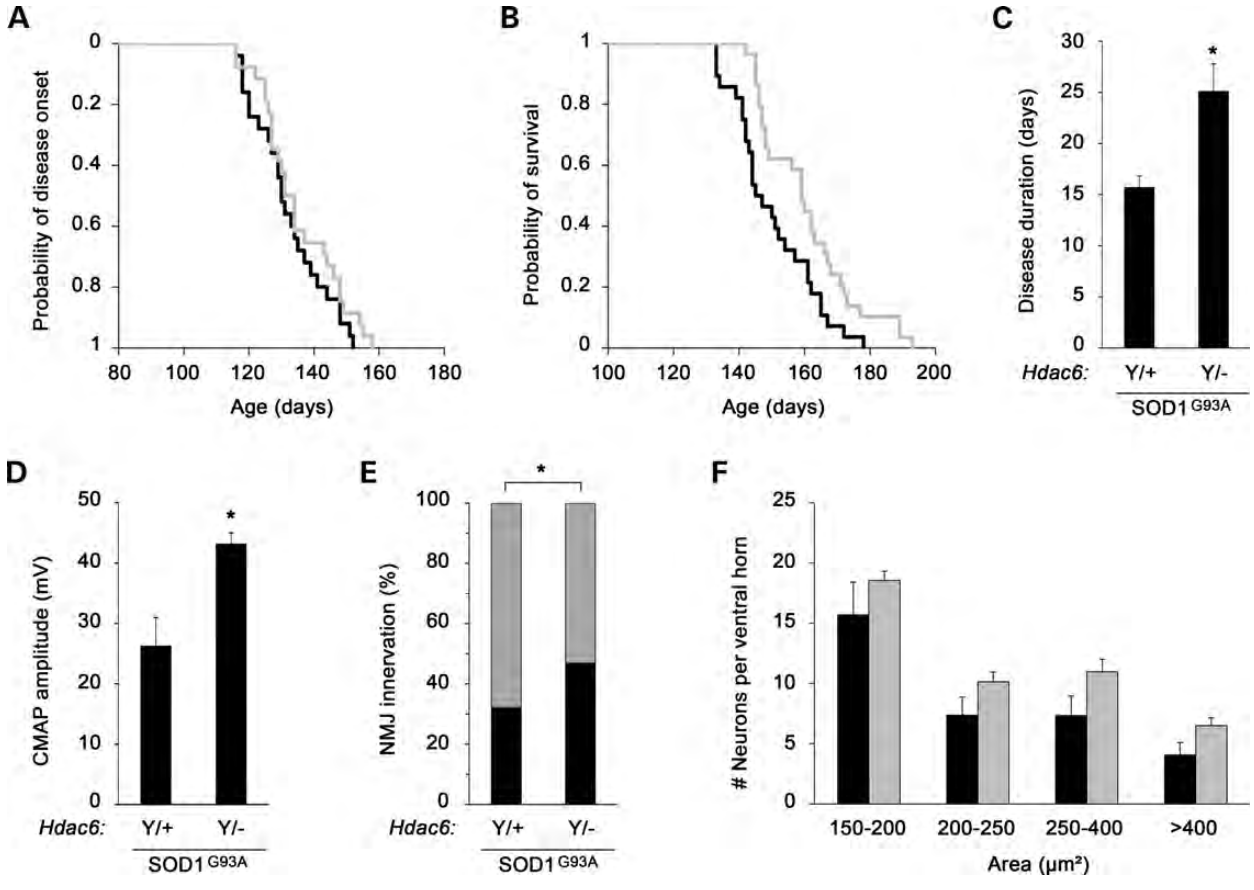

Figure 2. Effect of deletion of *Hdac6* on the course of motor neuron degeneration in $SOD1^{G93A}$ mice. (**A**) Probability of disease onset assessed by failure on the PaGE test ($SOD1^{G93A}/Hdac6^{Y/+}$ $n = 25$, black; $SOD1^{G93A}/Hdac6^{Y/-}$ $n = 26$, grey; log-rank, $P = 0.236$). (**B**) Probability of survival ($SOD1^{G93A}/Hdac6^{Y/+}$ $n = 28$, black; $SOD1^{G93A}/Hdac6^{Y/-}$ $n = 29$, grey; log-rank, $P = 0.008$). (**C**) Survival after disease onset ($SOD1^{G93A}/Hdac6^{Y/+}$ $n = 25$; $SOD1^{G93A}/Hdac6^{Y/-}$ $n = 26$; Student's *t*-test, $P = 0.003$). (**D**) CMAP amplitude of 130-day-old mice ($SOD1^{G93A}/Hdac6^{Y/+}$ $n = 7$; $SOD1^{G93A}/Hdac6^{Y/-}$ $n = 5$; Student's *t*–test, $^*P < 0.05$). (**E**) Proportion of complete (full innervation; black) and incomplete (partial or no innervation; grey) innervated NMJs in gastrocnemius muscle at 130 days of age [$n = 4$ per genotype; Chi-square test, OR = 0.535 (0.429–0.667), $^*P < 0.001$]. (**F**) Quantification of neurons in the ventral horn of 130-day-old $Hdac6^{+/+}$ (black) and $Hdac6^{-/-}$ (grey) mutant $SOD1^{G93A}$ mice categorized per size and normalized to 300 000 μm^2 of ventral horn ($n = 5$ per genotype; univariate ANOVA, $P = 0.006$ for genotype). (C, D and F) Data are represented as mean ± SEM.

$Hdac6^{Y/+}$ mice ($n = 28$) to 160 ± 3 days for $SOD1^{G93A}/Hdac6^{Y/-}$ mice ($n = 29$) ($P = 0.008$) (Fig. 2B). Median survival was increased from 145 days for $SOD1^{G93A}/Hdac6^{Y/+}$ mice to 159 days for Hdac6-deficient $SOD1^{G93A}$ mice. This effect may appear modest, but it should be noted that survival after disease onset was increased with 60% from 15.7 ± 1.1 days for $SOD1^{G93A}/Hdac6^{Y/+}$ to 25.1 ± 2.7 days for $SOD1^{G93A}/Hdac6^{Y/-}$ mice ($P = 0.003$) (Fig. 2C).

To confirm the survival benefit observed, we studied the CMAP in age-matched (130 days of age), symptomatic littermates, and quantified the innervation of their NMJs as well as the remaining neurons in the ventral spinal cord. CMAP amplitudes in $SOD1^{G93A}/Hdac6^{Y/-}$ mice were significantly higher compared with control $SOD1^{G93A}/Hdac6^{Y/+}$ mice (Fig. 2D, $P = 0.017$). Consistent with this increased function, NMJ innervation of the gastrocnemius muscle was better preserved in $SOD1^{G93A}/Hdac6^{Y/-}$ mice (47% complete innervation) compared with controls (32% complete innervation) [Fig. 2E, odds ratio (OR) = 0.535 (0.429–0.667), $P < 0.001$], and the number of remaining neurons in the ventral horn of $SOD1^{G93A}/Hdac6^{Y/-}$ mice was significantly higher than in $SOD1^{G93A}/Hdac6^{Y/+}$ mice (Fig. 2F, $P = 0.006$). This protective effect tended to be more pronounced for large neurons (>400 μm^2: +60%; 250–400 μm^2: +50%) than small neurons (200–250 μm^2: +38%; 150–200 μm^2: +18%).

The beneficial effects observed in the $SOD1^{G93A}/Hdac6^{Y/-}$ mice were associated with significantly increased levels of α-tubulin acetylation in the spinal cord ($P = 0.035$), sciatic nerve ($P = 0.021$) and brain ($P = 0.005$) (Fig. 3 and Supplementary Material, Fig. S3). The expression of the $SOD1^{G93A}$ transgene was not affected by the deletion of *Hdac6* and thus cannot account for the increase in survival of $SOD1^{G93A}/Hdac6^{Y/-}$ mice (Fig. 3A and Supplementary Material, Fig. S3A).

These results indicate that Hdac6 is a major α-tubulin deacetylase in the nervous system and that absence of Hdac6 does not induce neuromuscular abnormalities. Deletion of *Hdac6* from the $SOD1^{G93A}$ mouse significantly slowed the progression of motor neuron degeneration, suggesting that in this condition the beneficial effects of the absence of this enzyme (e.g. on axonal transport) outweigh its hazardous effects (e.g. on autophagy).

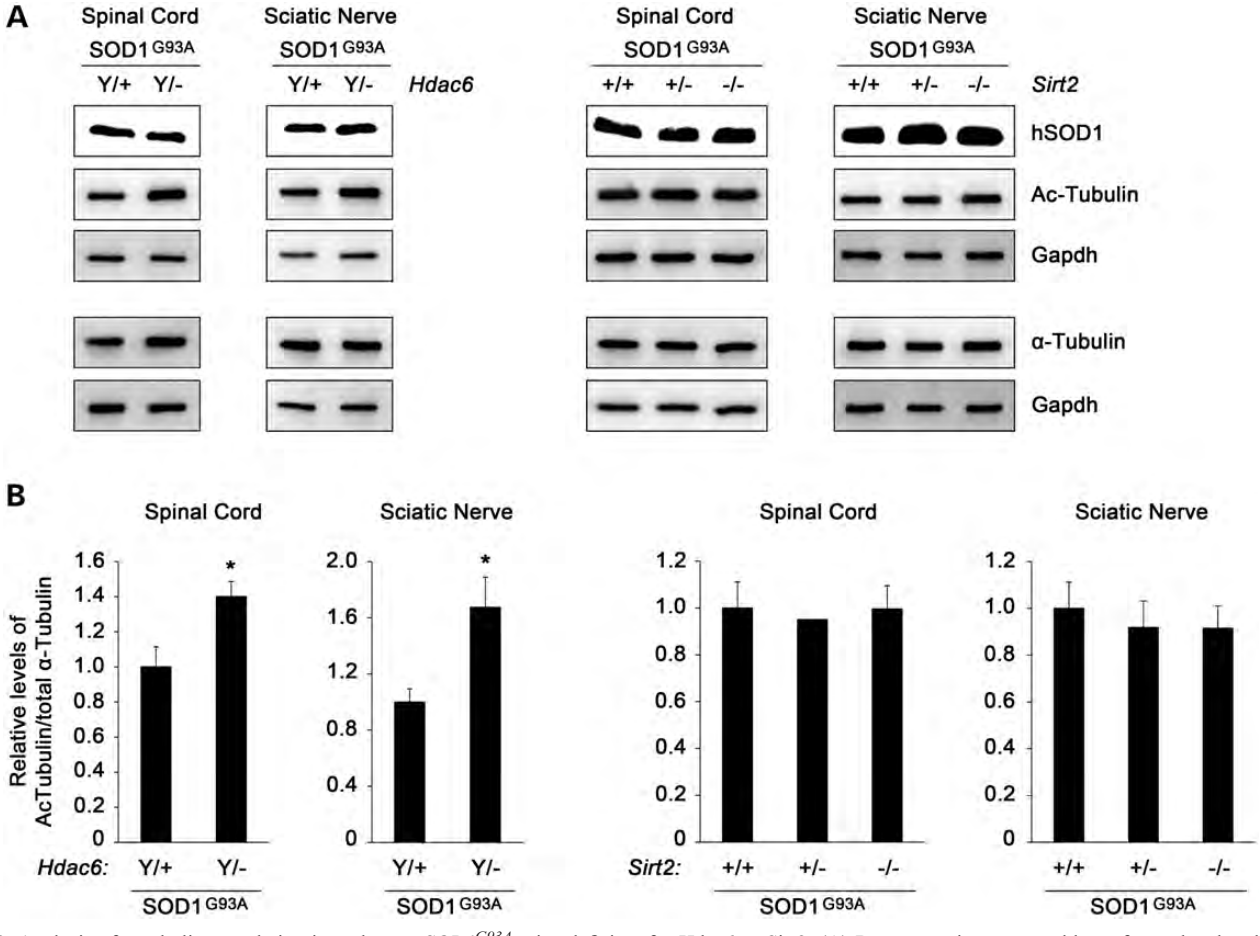

Figure 3. Analysis of α-tubulin acetylation in end-stage *SOD1*^{G93A} mice deficient for Hdac6 or Sirt2. (**A**) Representative western blots of acetylated and total α-tubulin levels in the nervous system. (**B**) Quantification of western blots. Levels of acetylated α-tubulin over total α-tubulin were normalized to Gapdh and shown as mean ± SEM. *SOD1*^{G93A}*/Hdac6* KO $n = 4–5$ per tissue and genotype; Student's *t*-test, $^{*}P < 0.05$. *SOD1*^{G93A}*/Sirt2* KO $n = 2–4$ per tissue and genotype; One-way ANOVA, spinal cord $P = 0.954$, sciatic nerve $P = 0.821$.

Effect of deletion of *Sirt2* on the course of motor neuron degeneration in *SOD1*^{G93A} mice

To study the effect of *Sirt2* deletion, we crossed *SOD1*^{G93A} mice with *Sirt2* KO mice. In contrast to what was observed for Hdac6, *Sirt2* deletion did not affect disease onset or survival of *SOD1*^{G93A} mice (Fig. 4A and B). In line with this lack of effect on behavioral disease parameters, we did not find any difference in CMAP amplitude or number of remaining neurons in the ventral spinal cord of symptomatic *SOD1*^{G93A}*/Sirt2*$^{+/+}$ mice and *SOD1*^{G93A} mice deficient for Sirt2 (Fig. 4C and D). Reduction of Sirt2 did not change SOD1^{G93A} expression (Fig. 3A and Supplementary Material, Fig. S3A).

To further explore the discrepancy between the effects of *Hdac6* KO versus *Sirt2* KO, we studied the effect of *Sirt2* deletion on the acetylation of α-tubulin in end-stage *SOD1*^{G93A} mice. In contrast to what we found in Hdac6-deficient mice, we did not find any difference in the level of acetylated α-tubulin upon deletion of *Sirt2* (Fig. 3 and Supplementary Material, Fig. S3). We confirmed this lack of effect at an earlier disease stage in age-matched (160 days of age) symptomatic littermates (Supplementary Material, Fig. S4). To exclude a confounding effect of the presence of SOD1^{G93A} and to elucidate whether Sirt2 actually regulates α-tubulin acetylation in the nervous system at all, we analyzed α-tubulin acetylation in spinal cord, brain and sciatic nerve of *Sirt2* KO mice. Surprisingly, but consistent with the results obtained, no differences in α-tubulin acetylation were present (Supplementary Material, Fig. S5). To exclude that the absence of Sirt2 was compensated for by upregulation of Hdac6, we quantified Hdac6 expression in 80-day-old and 250-day-old *Sirt2* KO mice, but found no alteration of Hdac6 mRNA in spinal cord and brain (Supplementary Material, Fig. S6).

These data suggest that although Sirt2 is clearly expressed in the nervous system (Fig. 1A), its contribution to the regulation of α-tubulin acetylation in this tissue is minor. Deletion of *Sirt2* does not induce neuromuscular abnormalities on itself and also fails to modify the disease course in the *SOD1*^{G93A} mouse.

DISCUSSION

Inhibitors of Hdac6 and Sirt2 have been suggested to be potential treatments for neurodegenerative diseases as they increase

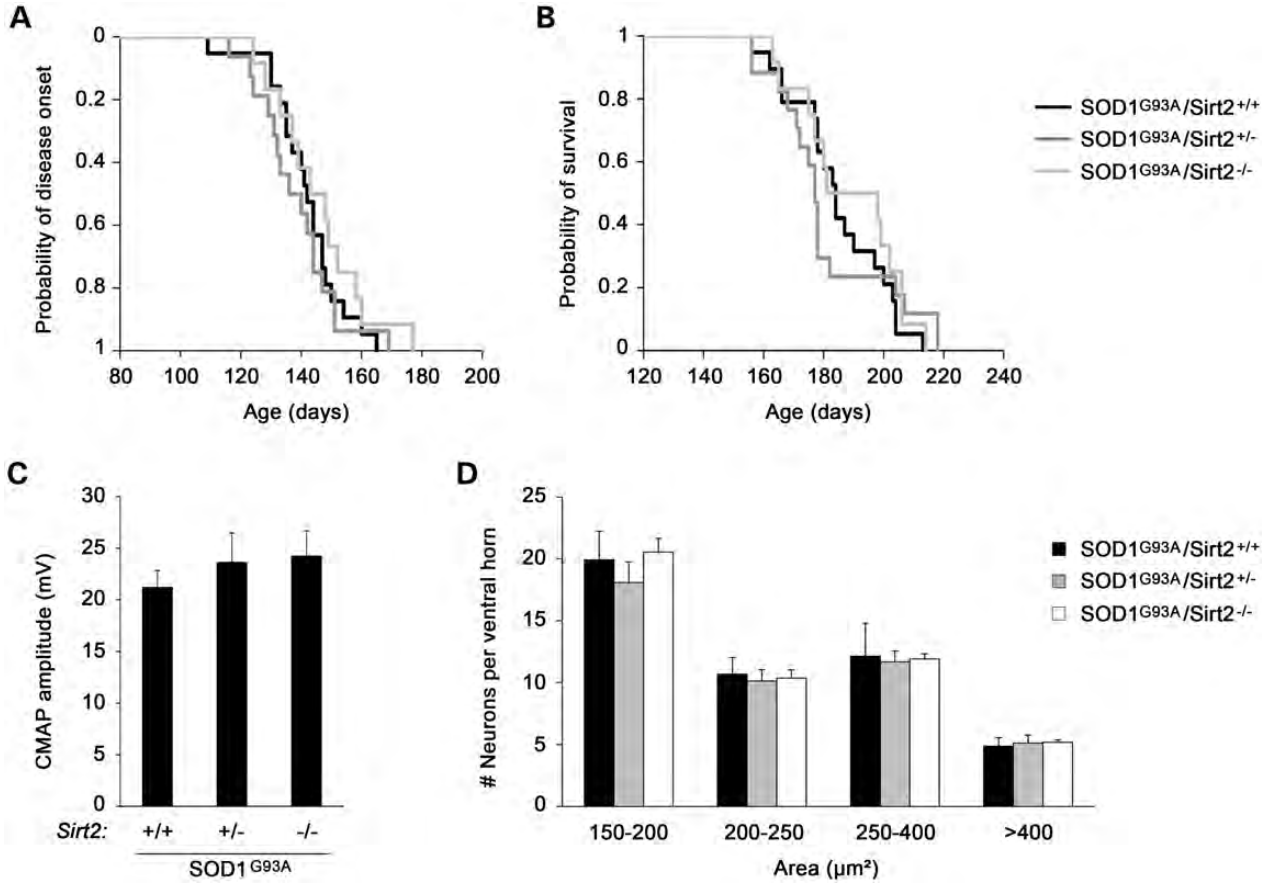

Figure 4. Effect of deletion of *Sirt2* on the course of motor neuron degeneration in $SOD1^{G93A}$ mice. (**A**) Probability of disease onset in $Sirt2^{+/+}$, $Sirt2^{+/-}$ and $Sirt2^{-/-}$ mutant $SOD1^{G93A}$ mice assessed by failure on the PaGE test (log-rank, $P = 0.376$). (**B**) Probability of survival of double transgenic mice (log-rank, $P = 0.870$). (A, B) The numbers of $SOD1^{G93A}$ mice used were: $Sirt2^{+/+}$ $n = 19$, $Sirt2^{+/-}$ $n = 16$, $Sirt2^{-/-}$ $n = 12$. (**C**) CMAP amplitude of 160-day-old $SOD1^{G93A}$ mice ($Sirt2^{+/+}$ $n = 7$; $Sirt2^{+/-}$ $n = 12$, $Sirt2^{-/-}$ $n = 8$; one-way ANOVA, $P = 0.737$). (**D**) Quantification of neurons in the ventral horn of the lumbar spinal cord categorized per size and normalized to 300 000 μm^2 of ventral horn of $Sirt2^{+/+}$, $Sirt2^{+/-}$ and $Sirt2^{-/-}$ mutant $SOD1^{G93A}$ mice at 160 days of age ($n = 3-4$ per genotype, univariate ANOVA, $P = 0.655$ for genotype). (C, D) Data are represented as mean $\pm$ SEM.

the acetylation of lysine at position 40 of α-tubulin. Increased acetylation at this site promotes the recruitment of the molecular motors kinesin-1 and cytoplasmic dynein (1,2), and facilitates intracellular trafficking and axonal transport. Defects in axonal transport have been implicated in the pathogenesis of neurodegenerative disorders characterized by the accumulation of misfolded protein [reviewed in (20)]. Protein accumulations can arise from deregulated transport, and in turn block the transport of other proteins, organelles and vesicles. Hence, improving axonal transport by inhibiting α-tubulin deacetylation is thought to be a strategy to treat these diseases. Genetic and pharmacological inhibitions of Hdac6 and Sirt2 have been studied in a variety of neurodegenerative disorders, such as HD, PD, Alzheimer's disease (AD), spinal and bulbar muscular atrophy (SBMA) and CMT (1,3,21–23), but these studies have generated contradictory results. In models of AD and SBMA, Hdac6 *activation* rather than inhibition was beneficial, (22,23). In contrast, in a model of HD, Hdac6 *inhibition* rescued the impaired transport of the brain-derived neurotrophic factor (BDNF) in striatal neurons *in vitro* (1). However, in an *in vivo* mouse model of HD, genetic deletion of *Hdac6* failed to modify disease progression or BDNF transport (24). In a CMT mouse model, Hdac6 inhibition reversed the phenotype *in vivo* and rescued the axonal transport defects (3). In cellular and *Drosophila* models of PD and HD, inhibition of Sirt2 was reported to have neuroprotective effects (4–6). These variable results may be explained by the fact that these enzymes have several different functions and several different substrates in the cell. This is particularly true for Hdac6, which plays an important role in autophagy through its ubiquitin-binding ability (8,9). The involvement of this enzyme in both axonal transport and misfolded protein degradation argues for a dual role of this protein in neurodegeneration. Depending on the type of neurodegeneration and perhaps even the disease stage, the importance of enhanced autophagy may outweigh the importance of restored axonal transport.

Ever since axonal swellings—resulting from neurofilament accumulations— have been observed in familial and sporadic ALS (14,15), defects in axonal transport are intensively studied in ALS. In order to elucidate whether increasing axonal transport through α-tubulin deacetylase inhibitors may

be a promising avenue for intervention in this disease, we investigated the effect of *Hdac6* and *Sirt2* deletion on the motor neuron degeneration in the *SOD1*^{G93A} mouse model of ALS.

The absence of the Hdac6 protein did not result in a neuromuscular phenotype on behavioral and physiological levels. This is consistent with previous findings of Bobrowska *et al.*, who tested motor coordination and balance, forelimb grip strength and spontaneous motor activity of *Hdac6* KO mice, and found no difference with control mice (24). Interestingly, no differences were observed in aggregate load of mutant huntingtin, suggesting that the role of Hdac6 in huntingtin aggregate clearance is minor or compensated by another protein. Although genetic depletion of Hdac6 indeed resulted in highly increased levels of acetylated α-tubulin, this did not modify behavioral phenotypes in HD mice. In contrast, we found that the absence of Hdac6 successfully modified the disease course in the *SOD1*^{G93A} mouse. *Hdac6* deletion did not affect disease onset, but significantly extended the survival after disease onset with as much as 60%. This increase in survival after disease onset is relevant for translation to human ALS, as treatment in patients is initiated after disease onset only.

As deletion of *Hdac6* from the *SOD1*^{G93A} mouse significantly slowed the progression of motor neuron degeneration, these results suggest that in this condition the beneficial effects of the absence of this enzyme (e.g. on axonal transport) are greater than its hazardous effects (e.g. on autophagy). This is in contrast to what has been described for models of HD, AD and SBMA (22–24). The benefit of Hdac6 activation rather than inhibition may point towards a more important role of impaired autophagy in the pathological mechanism of these disorders. Hdac6 inhibition was also beneficial in a model for motor neuropathy, most probably by increasing axonal transport through increased levels of acetylated α-tubulin (3). It would be interesting to verify whether the axonal transport in *SOD1*^{G93A} mice increases upon deletion of *Hdac6*. Unfortunately, measurement of axonal transport in adult mouse motor neurons *in vitro* is not feasible, in contrast to what can be done in sensory neurons (3), and techniques for measuring axonal transport *in vivo* are still being developed (12).

The absence of the other tubulin deacetylating enzyme, Sirt2, did not induce neuromuscular abnormalities, but did not modify the motor neuron degeneration in the mutant *SOD1* mice either. Surprisingly, we found that although Sirt2 is clearly expressed in the nervous system, its contribution to the regulation of α-tubulin acetylation in this tissue is minor. These results were confirmed in the absence of SOD1^{G93A} in order to exclude a confounding effect of the presence of SOD1^{G93A}. This is in contrast to what is generally accepted (25,26), but in agreement with the *in vivo* findings of Bobrowska *et al.* (27). Obviously, our data do not exclude that Sirt2 has a subtle effect on acetylation in certain cellular compartments that escape detection by western blot on the total tissue lysate, e.g. the oligodendrocyte, in which Sirt2 is highly expressed (28). But even then, our data show that deletion of *Sirt2* from those compartments does not affect motor neuron degeneration in the *SOD1*^{G93A} mouse, in spite of the fact that oligodendrocytes have been shown to contribute to neurodegeneration (29). Furthermore, as Sirt2 has been recently shown to play a pivotal role in programmed necrosis (7), our data indicate that this type of cell death does not contribute to the process of neurodegeneration such as seen in the *SOD1*^{G93A} model of ALS.

In conclusion, these findings suggest that inhibition of Sirt2 does not provide beneficial effect on ALS in rodents *in vivo*, in contrast to what has been reported in *in vitro* and *Drosophila* models of PD (5) and HD (4,6). In contrast, Hdac6 inhibition may be an appealing therapeutic strategy in ALS, as deletion of *Hdac6* attenuates motor neuron degeneration in the *SOD1*^{G93A} mouse. This attenuation is associated with an increase of acetylated α-tubulin, suggesting that its protective mechanism is related to changes in intracellular transport.

METHODS

Additional information is provided in Supplementary Methods.

Transgenic mice

Sirt2 KO mice, obtained by mating conditional *Sirt2* KO mice (16) with CMV-Cre mice, and *Hdac6* KO mice (17) were maintained in a C57Bl6/J background. Mice overexpressing human SOD1^{G93A} [B6SJL-TgN(SOD1-G93A)1Gur; The Jackson Laboratory] were crossed into a C57Bl6 background for more than 20 generations. All animals were housed under standard conditions according to the guidelines of the University of Leuven.

Disease onset and survival

To determine disease onset in *SOD1*^{G93A} mice, motor performance was evaluated twice every week by the PaGE test. For this, mice were placed on a wire grid upside down until they drop or reach the maximum of 60 s. Failure on the PaGE test was designated by dropping of the mouse before 60 s. Three *SOD1*^{G93A}/*Hdac6*$^{Y/+}$ mice and three *SOD1*^{G93A}/*Hdac6*$^{Y/-}$ mice were excluded from this test based upon poor initial performance, i.e. failure within the first 4 weeks of scoring. Survival was determined by loss of the righting reflex within 30 s. When this was the case, the mouse was sacrificed and this time point (equal to end stage) was considered as the time of death. Both the disease onset and survival are presented as Kaplan–Meier curves. For all the experiments, the investigator was blinded for the genotype.

Nerve conduction study

Mice were anesthetized with isoflurane and placed on a heating pad to maintain body temperature. Nerve conduction was measured using sub-dermal needle electrodes (Technomed Europe) and a Medelec EMG monitor (Medelec Vickers/Modul USA). To measure the compound muscle action potential (CMAP), the stimulating electrode was placed at the sciatic notch and the recording electrode at the level of the gastrocnemius muscle.

Histological analyses

Mice were sacrificed using CO_2 immediately followed by dissection of the gastrocnemius muscle, which was instantly

frozen in isopentane cooled with liquid nitrogen. To determine NMJ innervation, 40 μm thick longitudinal sections were stained with Alexa 555 conjugated α-bungarotoxin (1/500; Invitrogen) and rabbit anti-NF200 (1/200; Sigma) to visualize the axon innervating the NMJ. More than 100 NMJs spread over three to four sections of the gastrocnemius muscle were scored per animal. After transcardiac perfusion with phosphate buffered saline (PBS) and PBS with 4% paraformaldehyde (PFA), the lumbar region of the spinal cord was dissected, further fixed with 4% PFA, dehydrated in 30% sucrose solution and embedded in OCT medium (VWR). Sixty cryosections of 20 μm thickness each were made. Every sixth cryosection was stained with cresyl violet (Sigma) and used for quantitative analysis of the number of neurons. Thus, in total 10 sections were analyzed per spinal cord covering a range of 1.2 mm. At ×10 magnification, the area of normal appearing neurons with nucleoli in the ventral horn was calculated using AxioVision (version 4.8, Carl Zeiss) and the number of neurons in different size groups was determined.

Statistics

Analyses were performed using SPSS 16.0.2 software. Log-rank was used to analyze survival and disease onset; data from accelerating rotarod were analyzed by repeated measures ANOVA. Univariate ANOVA was applied for neuron counts. Student's *t*-test or one-way ANOVA was used for analyses of nerve conduction, disease duration, mRNA expression levels and protein expression levels. Chi-square test Pearson uncorrected was used to analyze the frequencies of NMJ denervation. α was a priori set at 0.05. Bonferroni's correction was applied in cases of multiple comparisons.

Study approval

All animal experiments were approved by the local Ethical Committee of the University of Leuven, Belgium (P020/2010).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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