Heterozygous Tbk1 loss has opposing effects in early and late stages of ALS in mice

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Heterozygous loss-of-function mutations of TANK-binding kinase 1 (Tbk1) cause familial ALS, yet downstream mechanisms of Tbk1 mutations remained elusive. Tbk1 is a pleiotropic kinase involved in the regulation of selective autophagy and inflammation. We show that heterozygous Tbk1 deletion alone does not lead to signs of motoneuron degeneration or disturbed autophagy in mice during a 200-d observation period. Surprisingly, however, hemizygous deletion of Tbk1 inversely modulates early and late disease phases in mice additionally overexpressing ALS-linked SOD1G93A, which represents a “second hit” that induces both neuroinflammation and proteostatic dysregulation. At the early stage, heterozygous Tbk1 deletion impairs autophagy in motoneurons and preproves both the clinical onset and muscular denervation in SOD1G93A/Tbk1+/− mice. At the late disease stage, however, it significantly alleviates microglial neuroinflammation, decelerates disease progression, and extends survival. Our results indicate a profound effect of Tbk1 on brain inflammatory cells under pro-inflammatory conditions and point to a complex, two-edged role of Tbk1 in SOD1-linked ALS.

Introduction

Some 5–10% of patients with the fatal neurodegenerative motor neuron (MN) disease amyotrophic lateral sclerosis (ALS) self-report a positive family history (familial ALS [fALS]). Using whole exome sequencing of fALS patients, we and others recently found an exome-wide, highly significant enrichment of mono-allelic TANK-binding kinase 1 (Tbk1) loss-of-function mutations in fALS and frontotemporal dementia (FTD) patients (Cirulli et al., 2015; Freischmidt et al., 2015).

Tbk1 is a pleiotropic kinase consisting of a catalytic kinase domain and three accessory regulatory domains. Its best characterized biological functions are the regulation of selective autophagy (Weidberg and Elazar, 2011; Wild et al., 2011; Pilli et al., 2012) and modulation of IFN signaling and inflammatory responses (Hemmi et al., 2004; Jin et al., 2012; Hasan et al., 2015, 2017; Yu et al., 2015). In addition, Tbk1 has been implicated in energy metabolism (Reilly et al., 2013, 2015; Everts et al., 2013; Zhao et al., 2018), microtubule dynamics (Pilli et al., 2015), and tumorigenesis (Ou et al., 2011).

Pointing to impaired autophagy and consequently reduced clearance of pathological protein oligomers/aggregates, brains of ALS/FTD patients with pathogenic Tbk1 mutations exhibit cytoplasmic (p)TDP-43- and p62-positive inclusions (Freischmidt et al., 2015; Pottier et al., 2015). Mouse studies with deletion of different autophagy-linked Atg genes indicate an ambiguous role of autophagy in MNs (Hara et al., 2006; Nassif et al., 2014; Tokuda et al., 2016; Rudnick et al., 2017).

Neuroinflammation, including activation of microglia and astrocytes, substantially contributes to the exacerbation and progression of the disease in mutant human SOD1 transgenic mouse models of ALS (Beers et al., 2006; Boillée et al., 2006; Yamanaka et al., 2008) and most likely in patients. Further, heterozygous deletion of the α-IFN receptor Ifnar1 significantly prolongs the life span of SOD1G93A mice (Wang et al., 2011). Intriguingly, Tbk1 is a well-known inducer of the IFN type I response (Trinchieri, 2010; Ahmad et al., 2016). By contrast, global heterozygous deletion of Tbk1 in combination with selective heterozygous deficiency of Takt in the myeloid lineage was recently shown to cause cortical neurodegeneration, microgliosis, and TDP-43 inclusions in 6-mo-old mice (Xu et al., 2018).

Taken together, Tbk1 is a central regulator of both selective autophagy and inflammatory responses via IFN type I signaling. Both pathways are suggested to influence the disease course of...
human ALS and have been shown to modulate disease in transgenic ALS mouse models. Consequently, our study sought to answer which pathways downstream of Tbk1 haploinsufficiency are the most ALS relevant.

**Results and discussion**

We aimed to determine a possible neurological phenotype of heterozygous Tbk1 knockout mice (Tbk1+/− mice). While homozygous loss of Tbk1 is embryonically lethal in mice (Bonnard et al., 2000), loss of one Tbk1 allele mirrors the genetic defect causing ALS/FTD in humans. In addition, we asked if and how a ubiquitous heterozygous Tbk1 knockout alters the phenotype of SOD1G93A transgenic mice. In this ALS model, the overexpression of (human) SOD1G93A leads to MN degeneration and neuroinflammation and represents a challenge of the proteostatic system (Phillips and Rothstein, 2015; Picher-Martel et al., 2016). Thus, we crossed Tbk1+/− mice with SOD1G93A transgenic mice. All four resulting genotypes (WT, Tbk1+/−, SOD1G93A, and SOD1G93A/Tbk1+/− siblings) were subjected to weekly rota rod testing, as well as assessment of the global phenotype progression, weight, and survival. Tbk1+/− and SOD1G93A/Tbk1+/− mice were phenotypically indistinguishable from WT or SOD1G93A siblings at birth. Tbk1+/− mice did not develop motor symptoms or experience weight loss or premature death during the study period of 200 d (Fig. 1, A–C). As expected, SOD1G93A mice developed hind limb tremor (clinical score of 1, see methods section), which became apparent to a blinded investigator at a mean age of 111.8 ± 3.3 d. Remarkably, heterozygous knockout of Tbk1 in addition to SOD1G93A overexpression (SOD1G93A/Tbk1+/− mice) preponed the onset of hind limb tremor to 99.1 ± 3.1 d (Δ12.6 d; P = 0.012; Fig. 1, A and D).

However, the age of onset of manifest gait disturbance (score of 2), peak weight, and peak rota rod performance did not significantly differ between SOD1G93A/Tbk1+/− and SOD1G93A siblings (Fig. 1, A–C and E; and Fig. S1, B), suggesting an attenuated progression of symptoms in SOD1G93A/Tbk1+/− mice. During the later disease course, SOD1G93A/Tbk1+/− mice indeed showed a further slowed decline in clinical score, weight, and rota rod performance when compared with SOD1G93A siblings (Fig. 1, A–C; and Fig. S1, A and C; SOD1G93A vs. SOD1G93A/Tbk1+/−: 10% weight loss: 148.5 ± 2.3 vs. 154.9 ± 1.5 d; Δ6.4 d; P = 0.044; half-maximal latency to fall in the rota rod test: 138.9 ± 2.6 vs. 142.3 ± 5.4 d; Δ3.4 d; P = 0.018). Finally, this also led to a significantly extended survival and overall disease duration of SOD1G93A mice with heterozygous Tbk1 knockout compared with single transgenic SOD1G93A siblings (despite the earlier appearance of first symptoms; Fig. 1 F and Fig. S1 D; SOD1G93A vs. SOD1G93A/Tbk1+/−: survival, 165.2 ± 3.7 vs. 184.2 ± 2.8 d; Δ19.0 d; P = 0.005; duration, 44.8 ± 3.0 vs. 63.9 ± 3.4 d; Δ19.1 d; P < 0.001).

Considering the unexpected, inverse effect of heterozygous Tbk1 knockout on clinical onset and disease course of SOD1G93A transgenic mice, we next sought to validate this observation at histological and biochemical levels and determine its molecular and cell biological correlates.

First, we analyzed the innervation of neuromuscular junctions (NMJs) of the pretibial muscles at the age of postnatal day (P) 50 (presymptomatic) and P120 (early symptomatic) in all four genotypes. Consistent with the lack of clinical symptoms during the study period, Tbk1+/− mice did not show NMJ denervation or MN loss at either time point compared with WT siblings (Fig. 2, A–H). By contrast, SOD1G93A/Tbk1+/− mice exhibited a significantly higher denervation of NMJs at P50 than the other groups including SOD1G93A siblings (Fig. 2, A and B), matching the significantly preponed detection of first clinical symptoms, whereas the number of lumbar spinal cord (LSC) MNs was still equal in all genotypes at P50 (Fig. 2, C and D). MN denervation and MN loss did not differ between SOD1G93A/Tbk1+/− mice and SOD1G93A siblings at P120 (Fig. 2, E–H). Conclusively, the kinetics of NMJ denervation and MN degeneration of SOD1G93A/Tbk1+/− mice are in agreement with a preponed symptom onset but subsequently decelerated disease progression (Fig. 1).

Regulation of autophagy is one of the best established biological functions of TBK1. Similar to the results of our study based on the heterozygous knockout of Tbk1, MN-selective homozygous knockout of the autophagy protein Atg7 preponed the onset of NMJ denervation and hind limb tremor in SOD1G93A mice (Rudnick et al., 2017). This indicates a possible neuroprotective effect of autophagy in the early disease of SOD1G93A transgenic mice. Hence, we sought to answer whether the heterozygous loss of Tbk1 impairs autophagy in LSC MNs in vitro and in vivo. We isolated primary MNs from the spinal cord of embryonic day (E) 12.5 WT and Tbk1+/− embryos using immunopanning (Wiese et al., 2010). Although we did not detect a central nervous system (CNS) phenotype in Tbk1+/− mice during the observation period of 200 d, embryonic Tbk1+/− MNs show impaired axonal elongation after 7 d in culture (Fig. 3, A and B), which points to a possible role of Tbk1 in axon growth during development. Alternatively, the observation reflects an MN deficit demasked by cell culture stress as a “second hit,” analogous to the (accen tuated) Tbk1-associated axon terminal/NMJ phenotype we observed only in SOD1G93A/Tbk1+/− double-mutant mice compared with SOD1G93A mice (Fig. 2, A and B).

Furthermore, we observed significantly increased levels of p62, the Tbk1-binding autophagy adaptor protein optineurin (OPTN) and LC3-II in Tbk1+/− primary MNs after 7 d in culture, suggesting an impairment of autophagy (Fig. 3, C and D). SOD1 and TDP-43 levels were not affected by Tbk1 haploinsufficiency (Fig. 3, C and D).

Consequently, we extended our analysis of autophagy markers to the in vivo situation. LSC lysates and slices from P50 and P120 mice of all four genotypes were analyzed. The autophagy markers LC3–I, LC3–II, and p62 were unaltered at the age of P50 and P120 in all four genotypes, according to Western blotting of spinal cord protein lysates (Fig. S2, A–D). However, single cell–based analysis using immunohistochemical staining revealed an increased accumulation of aggregated p62 forming round bodies in the cytoplasm of LSC MNs of SOD1G93A/Tbk1+/− mice at P50 compared with SOD1G93A siblings, while Tbk1+/− and WT mice showed essentially no p62+ inclusions (Fig. 3, E and F). We could reproduce this finding using an alternative autophagy marker, GABARAPL1, an Atg8 homologue highly expressed in MNs (Le Grand et al., 2013; Fig. 3, E and G).

As shown in Fig. S2, G and H, the mostly round p62+ inclusions in both SOD1G93A transgenic groups largely colocalized with

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GABARAPL1 as well as polyubiquitin at P50. We hardly detected round p62 inclusions within ChAT+ neurons at P120, most probably because the majority of large MNs containing these round p62 inclusions at P50 belonged to the more susceptible MN subpopulation and had degenerated between P50 and P120 (as also described previously; e.g., Rudnick et al., 2017). Instead of round p62 inclusions the remaining smaller neurons partially exhibited “skein-like” p62 inclusions at P120 (Fig. S2, K and arrowhead in M), which is consistent with previous findings (Rudnick et al., 2017). The majority of p62 aggregates, however, were not located inside but were outside of the MN somata at P120 and overlapped with GABARAPL1 and polyubiquitin (Fig. S2, I and J). Co-staining of Nissl, Neurofilament, GFAP and Iba1 with p62 suggested that the p62 aggregates outside MN somata colocalized with axons and astrocytes, but hardly with microglia (Fig. S2, K–M). Quantitative analyses revealed that the levels of aggregated p62 inside the remaining ChAT+ MNs at P120 differed significantly between non-SOD1G93A-transgenic and SOD1G93A mice, but not between SOD1G93A mice and SOD1G93A/Tbk1+/− mice, despite a nonsignificant trend toward a reduction in SOD1G93A/Tbk1+/− mice (P = 0.19; Fig. 3H).

Taken together, the above evidence suggests that at P120 the decelerated disease progression starts to outmatch the disadvantage of Tbk1 haploinsufficiency observed at P50.

TBK1 and phospho-S177–OPTN colocalize with SOD1G93A aggregates in MNs of SOD1G93A mice, suggesting that SOD1G93A aggregates undergo TBK1-directed selective autophagy (Korac et al., 2013). Pointing in the same direction, heterozygous deletion of the autophagy protein Becn1 (Atg6) enhances aggregation of mutant SOD1G93A in SOD1G93A mice (Tokuda et al., 2016). However, we did not find differences in the quantity of either endogenous mouse (WT) SOD1 or human misfolded SOD1G93A in LSC by Western blotting or immunohistochemistry between mutant SOD1G93A mice with or without heterozygous Tbk1 knockout (Fig. 3, I–K; and Fig. S2, E and F). This indicates that the prepone onset of motor symptoms and NMJ denervation in SOD1G93A/Tbk1+/− mice is unlikely to be a direct consequence of increased SOD1G93A levels and suggests other proteins or organelles to be the relevant substrates of disturbed autophagy that are responsible for the effect of heterozygous Tbk1 knockout in the SOD1G93A model.

Beyond its role as an autophagy regulator, abundant evidence connects TBK1 to inflammation. TBK1 is a well-known inducer of the IFN type I response (Trinchieri, 2010). Notably, heterozygous deletion of Ifnar1 significantly prolongs the life span of SOD1G93A mice (Wang et al., 2011) to an extent similar to that observed in the SOD1G93A/Tbk1+/− mice of this study. Moreover, reactive astrocytes and microglia have been repeatedly demonstrated to accelerate disease progression in mutant human

Figure 1. Heterozygous Tbk1 deletion prepones early motor symptoms but slows disease progression and prolongs survival in the SOD1G93A ALS mouse model. (A) Progression of the clinical score at group level. SOD1G93A/Tbk1+/− mice show a bi-phasic, first accelerated and then slowed, disease progression compared with SOD1G93A siblings. (B) Weight curve at group level. SOD1G93A/Tbk1+/− mice show a slowed progression of weight loss compared with SOD1G93A siblings. (C) Performance in the rotarod test at group level over time. SOD1G93A/Tbk1+/− mice show a slowed progression of motor decline compared with SOD1G93A siblings. (D) Kaplan-Meier plot of the fraction of mice with hind limb tremor (score of 1). SOD1G93A/Tbk1+/− mice present with a significantly earlier onset of hind limb tremor than SOD1G93A siblings. (E) Kaplan-Meier plots of the fraction of mice having reached their weight peak. SOD1G93A/Tbk1+/− and SOD1G93A siblings exhibit a similar onset of weight loss. (F) As demonstrated by Kaplan-Meier survival curves, heterozygous deletion of Tbk1 significantly prolongs survival of SOD1G93A mice. n = 16–18 male mice per group in all graphs. Data in A–C are presented as means ± SEM and were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons post hoc test. Kaplan-Meier plots were analyzed using the log-rank (Mantel-Cox) test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
SOD1 transgenic mice (Beers et al., 2006; Boillée et al., 2006; Yamanaka et al., 2008).

Hence, we hypothesized that Tbk1 haploinsufficiency, beyond autophagy regulation and its role in peripheral leukocytes, may also alleviate CNS inflammation and thereby extend the life span in the mutant SOD1 model. Consequently, we quantified the microglial and astrocytic response at the age of P120, when robust neuroinflammation is found in the SOD1G93A model. Of note, we did not detect differences in the abundances of microglia and astrocytes in the LSC between Tbk1+/− mice and WT mice. By contrast, we found a significantly alleviated microglial activation in SOD1G93A/Tbk1+/− mice compared with SOD1G93A siblings. Immunohistochemical analysis of the LSC of SOD1G93A/Tbk1+/− mice showed a smaller area and reduced integrated density of the Iba1 immunoreactivity, as well as a reduced mean Iba1+ cell size, compared with SOD1G93A siblings (Fig. 4, A–D). In addition, we observed a reduced immunopositive area of the microglial activation markers CD68 and Clec7a (Holtman et al., 2015;
Krasemann et al., 2017) in SOD1G93A/Tbk1+/− mice compared with SOD1G93A siblings (Fig. 4, A, E, and F). Clec7a has been described as a marker for the neurodegeneration-associated microglial profile in various models, including SOD1G93A mice (Holtman et al., 2015; Krasemann et al., 2017).

We furthermore observed a trend toward reduced astroglia- sis in the LSC of SOD1G93A/Tbk1+/− mice compared with SOD1G93A siblings at P120, based on GFAP area or integrated GFAP immunoreactivity (P = 0.17 and P = 0.52, respectively; Fig. 4, G–I). There was no microgliosis or astrogliosis detectable in LSCs in any of the four genotypes at P50 (Fig. S2, N–P).

To corroborate the results from these immunohistochemical studies, we next aimed to obtain a more comprehensive picture of neuroinflammation in vivo. To that end, we performed a quantitative NanoString nCounter mRNA expression profiling of a large inflammation-related gene panel (800 genes) of the spinal cord of symptomatic mice and control siblings at P140. In line with the behavioral and immunohistochemical experiments, only the expression of 1 of 800 genes (Ceruloplasmin; [Cp]), beyond Tbk1 itself (which was reduced due to loss of one gene copy), was significantly altered in Tbk1+/− animals compared with WT mice after correction for multiple testing. Consistent with the known strong inflammatory response in ALS mice, we measured 542 inflammation related genes to be significantly altered in SOD1G93A mice compared with WT siblings (mostly induced or increased expression). Upon heterozygous Tbk1 deletion, 96 inflammation-related genes showed significantly less strong induction (among them, the microglial neurodegenerative phenotype markers Axl, Lilrb4, Clec7a, Csfl, and Trem2, previously described in Krasemann et al., 2017), indicating an attenuated inflammatory response in SOD1G93A/Tbk1+/− mice (supplemental dataset). Further supporting that Tbk1 haploinsufficiency attenuates the pro-inflammatory effect of the mutant SOD1 transgene, an unbiased hierarchical cluster analysis did not separate Tbk1+/− from WT animals, but completely separated SOD1G93A/Tbk1+/− mice from their SOD1G93A siblings (Fig. S5 A).

Furthermore, the comprehensive NanoString nCounter mRNA expression dataset allowed us to estimate the cell type-specific marker gene signatures described by Danaher et al. (2017) in the analyzed spinal cord tissue. This analysis revealed a significantly attenuated expression of inflammatory genes normally associated with microglial and/or astrocytic responses in SOD1G93A/Tbk1+/− compared with SOD1G93A siblings (Fig. 5 B). Endothelial cell type marker analysis served as a negative control and did not differ among the four genotypes (Fig. 5 B).

Overall, our results show that a global heterozygous deletion of Tbk1 reduces the neuroinflammatory response and attenuates the neurodegeneration-associated differentiation profile of microglia in SOD1G93A mice. To address the question of whether Tbk1 haploinsufficiency has a direct impact on microglial activation, we analyzed the mRNA levels of a subset of genes representing microglial activation markers and/or Tbk1 downstream targets in purified primary microglia cultures. Consistent with our in vivo data, several classically rather pro-inflammatory genes (e.g., Ccl4, Il1b, Nos2) were less induced in LPS-treated Tbk1+/− compared with WT microglial cells, pointing to an attenuated inflammatory response in vitro (Fig. 5 C). Under the cell culture condition without LPS stimulation, which nevertheless represents a less physiological situation than in vivo, there were also some changes in neuroinflammatory gene expression due to heterozygous Tbk1 deletion (Fig. 5 C). Our in vivo results support the hypothesis that microglia are among the primary cell types mediating the modulation of inflammation by Tbk1 haploinsufficiency in the spinal cord of ALS mice.

Taken together, we provide evidence that TBK1 has opposing effects in the early and late phases of disease in the mutant SOD1G93A mouse model of ALS. At the early stage, heterozygous Tbk1 deletion impairs autophagy in MNs and prepones hind limb tremor and NMJ denervation. At the late disease stage, heterozygous Tbk1 deletion substantially alleviates glial activation. Considering our in vivo and in vitro data, the attenuation of neuroinflammation represents the most likely mechanism responsible for the decreased disease progression rate and extended survival of SOD1G93A transgenic mice. In this context, the slowed disease progression observed in SOD1G93A/Tbk1+/− mice would thus represent a non–cell-autonomous effect, in full agreement with abundant previous evidence connecting neuroinflammation and disease progression in the mutant SOD1 ALS mouse model (Beers et al., 2006; Boillée et al., 2006; Yamanaka et al., 2008). However, given the pleiotropic nature of TBK1, multiple alternative cellular pathways may contribute to the alleviated progression and prolonged life span of the SOD1G93A transgenic mice. Cell-type selective deletion of Tbk1 in nonneuronal cells will be necessary for the final confirmation of this claim. In contrast, considering both our in vivo results and the effects of heterozygous Tbk1 deletion on purified cultured MNs, accelerated onset of symptoms and increased NMJ denervation in mutant SOD1G93A mice with heterozygous Tbk1 deletion are more likely MN-autonomous effects. Nevertheless, despite the abundant cell biological data linking TBK1 and autophagy, a caveat must be...
expressed because the loss of a Tbk1 allele could also indirectly lead to enhanced autophagy impairment in MNs, triggered, for example, by a primary damage to Schwann cells, muscle cells, or macrophages with subsequent damage to motor axons.

Although the heterozygous deletion of Tbk1 in mice corresponds exactly to the (haploinsufficient) Tbk1 loss-of-function mutations that are associated with ALS in humans, we could not detect a motoneuronal phenotype at the behavioral or histological level in Tbk1+/− mice compared with WT mice until the end of the study at an age of 200 d. Of note, in humans Tbk1 loss-of-function mutations show a reduced penetrance and somewhat later age at onset compared with most other fALS mutations (Freischmidt et al., 2015). Thus, we speculate that ALS-linked Tbk1 loss-of-function mutations may require one or more specific genetic or other cofactors representing “second hits” that are present in only a fraction of the normal population and not in mice. This hypothesis is in agreement with our observation that only a second genetic stressor, i.e., mutant SOD1 overexpression, demasks the effects of heterozygous Tbk1 deletion in the CNS of mice.

**Figure 4.** Tbk1 haploinsufficiency mitigates microglial neuroinflammation in SOD1G93A mice. (A) Representative photomicrographs of LSC hemispheres stained for the microglia markers Iba1, CD68, and Clec7a. Scale bars, 100 µm; 50 µm in magnified images. (B–F) SOD1G93A/Tbk1+/− mice showed a reduced Iba1+ area, CD68+ area, and Clec7a+ area, reduced integrated density, and reduced mean size of Iba1+ microglia, compared with SOD1G93A siblings. (G) Representative photomicrographs of astroglia in the gray matter of the LSC at the age of P120. Scale bar, 100 µm. (H and I) A nonsignificant trend toward reduced GFAP+ area (I) and mean integrated density of GFAP (H) in SOD1G93A/Tbk1+/− mice compared with SOD1G93A siblings was observed. n = 5–7 female mice per group. Data are presented as means ± SEM. Data were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons post hoc test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
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In summary, our study demonstrates that a heterozygous deletion of Tbk1 is sufficient to affect the function of multiple CNS cell types—at least neurons, astrocytes, and microglia. Our study is thus an important basis for further research on the complex and, depending on the disease stage, antipodal roles of Tbk1 in ALS and other CNS disorders.

Material and methods

Animals

B6.Cg-Tg(SOD1*G93A)1Gur/J mice (stock no. 004435; high transgene copy number; https://www.jax.org/strain/004435; hereafter referred to as SOD1G93A mice) were purchased from Jackson Laboratories. Heterozygous B6.129P2-Tbk1tm1Aki mice (hereafter referred to as Tbk1+/− mice; http://www.informatics.jax.org/allele/key/29752; Hemmi et al., 2004) were kindly provided by Shizuo Akira. SOD1G93A mice were bred with Tbk1+/− mice to obtain SOD1G93A/Tbk1+/− mice and respective control siblings. Both strains were bred on the same C57Bl/6 genetic background, and only F1 offspring were analyzed. Tbk1+/− mice had been backcrossed with C57Bl/6j mice for >10 generations. Mice were maintained at 22°C with a 14/10-h light/dark cycle and had food and water ad libitum. All animal experiments were performed in accordance with institutional guidelines of the University of Ulm and were approved by the local authority (Regierungspräsidium Tübingen, Germany; animal permission no. 1253).

Genotyping

Genotyping of the SOD1G93A transgene was performed according to the Jackson Laboratory standard PCR protocol (https://www.jax.org/strain/004435). Genotyping of Tbk1-KO was performed as previously described (Möser et al., 2015).

Scoring and motor testing

Twice a week, male mice were subjected to weighing and disease scoring. The mice were evaluated for signs of motor deficit using the following five-stage point scoring system adapted from Weydt et al. (2003): stage 1, manifest hind limb tremor when suspended by the tail; stage 2, manifest gait abnormalities; stage 3, manifest paralysis of one hind limb; stage 4, manifest paralysis of both hind limbs; and stage 5, end stage. The onset (stage 1) was defined retrospectively as the earliest time when the mice showed symptoms for at least two consecutive weeks. The end-stage was determined as the inability to rise when suspended by the tail; stage 2, manifest gait abnormalities; stage 3, manifest paralysis of one hind limb; stage 4, manifest paralysis of both hind limbs; and stage 5, end stage. The latency to fall off the rotarod was tested weekly from P50 until end stage by a blinded experimenter using a Rotarod for five mice with tactile user interface (#LE8205; BIOSEB). Rotarod testing consisted of consecutive three rounds per mouse; only the maximum value per day was considered. The start speed of the rod was adjusted to 4 rpm and was accelerated to the maximum speed of 40 rpm during 300 s.

Tissue preparation

At the described time points, mice were deeply anesthetized by i.p. injection of a ketamine/Rompun mixture and were transcardially perfused with 20 ml PBS and 20 ml of 4% paraformaldehyde for fixation. Spinal cords and muscles were fixed overnight with 4% paraformaldehyde, then dehydrated in 30% sucrose (Sigma-Aldrich) in PBS for 48 h at 4°C, embedded in Tissue-Tek O.C.T. Compound (Sakura), and stored at −80°C until use. Embedded spinal cords were sectioned into 12-µm coronal slices using a cryotome (Leica). Serial sections covering the whole LSC were obtained from each animal. Every 10th section was chosen for quantification of anterior horn MNs (total eight sections). Every 20th section was used for quantification of microglia and astrocytes (total four sections each). Pretibial muscles were sectioned into 25-µm longitudinal slices. At least 300 NMJs were recorded per genotype. Mouse whose tissue was used for protein analysis were killed by decapitation. The extracted tissue was immediately transferred to liquid nitrogen and stored at −80°C until use.

Immunohistochemistry

Transverse sections of the spinal cord (12 µm thick) were cut using a cryotome. Sections were blocked for 1 h using a permeabilization/blocking solution containing Tris-buffered saline (TBS) with 5% FCS, and 0.25% Triton X-100 (Sigma-Aldrich). After washing once with TBS, sections were stained with combinations of mouse anti-NeuN (1:500; Millipore), goat anti-ChAT (1:1,000; Abcam), rabbit anti-GFAP (1:1,000; Abcam), α-bungarotoxin 488 (Invitrogen), mouse anti-p62 (1:500; Invitrogen), rabbit anti-p62 (1:2,000; MBL), rabbit anti-GABARAPL1 (1:1,000; Proteintech), mouse anti-polyubiquitin (1:500; Enzo), rat anti-Clec7a (1:30; InvivoGen), mouse anti-misfolded human SOD1 clone B8H10 (1:250; Medimabs), rabbit anti-Iba1 (1:500; Wako), goat anti-Iba1 (1:1,000; Abcam), rat anti-CD68 (1:100; Bio-Rad), rabbit anti-GFAP (1:750; Abcam), ab-bungarotoxin 488 (Invitrogen; 1:1,000), mouse anti-Neurofilament marker (SMI-31; 1:1,000; BioLegend), and anti-Synaptophysin (1:1,000; Abcam).

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Genotyping of the SOD1G93A transgene was performed according to the Jackson Laboratory standard PCR protocol (https://www.jax.org/strain/004435). Genotyping of Tbk1-KO was performed as previously described (Möser et al., 2015).

Scoring and motor testing

Twice a week, male mice were subjected to weighing and disease scoring. The mice were evaluated for signs of motor deficit using the following five-stage point scoring system adapted from Weydt et al. (2003): stage 1, manifest hind limb tremor when suspended by the tail; stage 2, manifest gait abnormalities; stage 3, manifest paralysis of one hind limb; stage 4, manifest paralysis of both hind limbs; and stage 5, end stage. The onset (stage 1) was defined retrospectively as the earliest time when the mice showed symptoms for at least two consecutive weeks. The end-stage was determined as the inability to rise within 30 s after being placed on its side. The latency to fall off the rotarod was tested weekly from P50 until end stage by a blinded experimenter using a Rotarod for five mice with tactile user interface (#LE8205; BIOSEB). Rotarod testing consisted of consecutive three rounds per mouse; only the maximum value per day was considered. The start speed of the rod was adjusted to 4 rpm and was accelerated to the maximum speed of 40 rpm during 300 s.
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Primary astrocyte and microglia culture

Primary cells were prepared from P1-4 *Tbk1<sup>−/−</sup>* pups and WT siblings. Microglia were prepared as previously described (Wiesner et al., 2013). Substances and solutions were from Gibco or Sigma-Aldrich. In brief, for microglia, forebrains were digested and dissociated. All cells were seeded in T75 cell culture flasks in supplemented DMEM (Gibco). After 7–10 d in culture, microglia were manually shaken off the astrocyte layer and seeded on 6-well plates with 6 × 10<sup>5</sup> cells. Microglia cells were harvested up to three times from the astrocyte co-culture. Subsequently, astrocytes were washed from remaining microglia cells and seeded on 6-well plates with 10<sup>5</sup> cells. Microglia were stimulated with LPS 50 ng/ml for 6 h.

Western blotting

Urea lysis buffer (8 M urea, 10 mM Tris, and 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0) and TissueLyser II (Qiagen) were used to extract protein from spinal cord tissue. Immunoblotting was performed according to standard procedures, using a total protein amount of 20–30 μg per sample and the XCell II Blot Module system (Thermo Fisher Scientific). The following antibodies were used: rabbit anti-GAPDH (1:1,000; Proteintech), rabbit anti-β-actin (1:1,000; Cell Signaling), rabbit anti-LC3B (1:1,000; Cell Signaling), rabbit anti-LC3B (1:1,000; MBL), mouse anti-SQSTM1/p62 (1:1,000; Abcam), rabbit anti-TBK1 (1:1,000; Thermo Fisher Scientific), rabbit anti-SOD1 (1:1,000, Enzo Life Science), goat anti-mouse-HRP (1:1,000, Life Technologies), and goat anti-rabbit-HRP (1:1,000, Life Technologies). Membranes were developed using the Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the FUSION SOLO S (Peglab) system.

Real-time PCR

Total RNA was isolated from primary glial cultures using the RNeasy Plus Micro kit (#74404; Qiagen). Reverse transcription reactions were performed with the Quantitect Reverse Transcription Kit (#205311; Qiagen), according to the manufacturer’s instructions. Subsequent PCR reactions were run in duplicate on a CFX96 real-time system (Bio-Rad), using the Quantitect SYBR Green PCR Kit (#204143; Qiagen) and Quantitect Primer Assays (Qiagen). The following Quantitect primer assays were used: Ywhaz (#Q070105350), Tbp (#Q070198443), C1qa (#Q101660778), Ccl2 (#Q070167832), Ccl3 (#Q070285426), Ccl4 (#Q070154615), Ilb1 (#Q01048335), Il6 (#Q070098875), Il10 (#Q070106169), Tnf (#Q070104006), Ifnγ (#Q070245262), Irf7 (#Q0701048698), Stat2 (#Q070160216), Isg15 (#Q07172876), Ifnβ (#Q070249662), Stat1 (#Q070162183), and Nos2 (#Q070100275). The resulting Ct values were normalized to two housekeeping genes (Ywhaz and Tbp) using the 2<sup>−ΔΔCt</sup> method (Livak and Schmittgen, 2001).

Quantitative NanoString nCounter mRNA expression analysis

Total RNA was isolated from spinal cords of P140 mice using the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer’s instructions. Multiplexed mRNA analysis of 800 genes (nCounter Mouse Neuroinflammation Panel plus 30 additional genes [mainly known ALS genes]) was performed using the nCounter platform. The Cell Type Profiling Module of the nCounter Advanced Analysis 2.0 plugin for the nSolver software
(NanoString) was used to measure the abundance of various cell populations according to Danaher et al. (2017). The method quantifies cell populations using marker genes that are expressed stably and specifically in given cell types (nCounter Advanced Analysis 2.0 User Manual MAN-10030-03).

Statistics

For comparison of multiple groups, the statistical significance of endpoints was evaluated by one-way ANOVA followed by Tukey’s multiple comparisons post hoc test when data were normally distributed. When data were not normally distributed, statistical significance of endpoints was evaluated by Kruskal-Wallis test followed by Dunn’s multiple comparisons post hoc test. For comparison of two groups and normal distribution of data, the unpaired two-tailed Student’s t test was used. Kaplan-Meier plots were analyzed using the log-rank (Mantel-Cox) test. See the analysis scheme in the supplemental dataset for statistical methods used for analysis of the NanoString nCounter mRNA data. Data are presented as means ± SEM in bar graphs. Statistical significance was reported by the P value of the statistical test procedures and was assessed as significant (*, P < 0.05), strongly significant (**, P < 0.01), or highly significant (***, P < 0.001; ****, P < 0.0001). All statistical analyses were performed with Prism software (version 7.02; GraphPad Software).

Online supplemental material

Fig. S1 shows Kaplan-Meier plots for weight loss and maximum as well as half-maximum latency to fall in the rotarod test and a bar graph displaying disease duration of SOD1G93A/Tbk1+/− compared with SOD1G93A mice. Fig. S2 shows analysis of protein levels of SOD1 and autophagy markers in spinal cord lysates and shows representative pictures of intra- and extramotorneural protein inclusions at P50 and P120. Furthermore, it shows quantification of astrogliosis and microglia at P50.

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