Gasdermin-E mediates mitochondrial damage in axons and neurodegeneration

Highlights

- GSDME is expressed by neurons and activated by mitochondrial toxins
- Neuronal GSDME drives local mitochondrial damage and axon loss prior to cell death
- ALS/FTD proteins activate GSDME and drive neurite loss in mouse and human neurons
- SOD1G93A GSDME KO mice have rescued disease progression and motor neuron loss

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In brief

Gasdermin-E is a pore-forming molecule that is expressed in neurons. When activated, this molecule rapidly targets and destroys mitochondria. In neurons, toxin exposure activates GSDME and drives mitochondrial and axonal destruction. The genetic inhibition of GSDME rescues axon loss in amyotrophic lateral sclerosis (ALS) patient-derived motor neurons and ameliorates disease in a mouse model of ALS.
**Article**

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**SUMMARY**

Mitochondrial dysfunction and axon loss are hallmarks of neurologic diseases. Gasdermin (GSDM) proteins are executioner pore-forming molecules that mediate cell death, yet their roles in the central nervous system (CNS) are not well understood. Here, we find that one GSDM family member, GSDME, is expressed by both mouse and human neurons. GSDME plays a role in mitochondrial damage and axon loss. Mitochondrial neurotoxins induced caspase-dependent GSDME cleavage and rapid localization to mitochondria in axons, where GSDME promoted mitochondrial depolarization, trafficking defects, and neurite retraction. Frontotemporal dementia (FTD)/amyotrophic lateral sclerosis (ALS)-associated proteins TDP-43 and PR-50 induced GSDME-mediated damage to mitochondria and neurite loss. GSDME knockdown protected against neurite loss in ALS patient iPSC-derived motor neurons. Knockout of GSDME in SOD1<sup>G93A</sup> ALS mice prolonged survival, ameliorated motor dysfunction, rescued motor neuron loss, and reduced neuroinflammation. We identify GSDME as an executioner of neuronal mitochondrial dysfunction that may contribute to neurodegeneration.

**INTRODUCTION**

Neurologic diseases are often characterized by early mitochondrial dysfunction and axon loss preceding cell death.1-4 Mitochondrial damage leads to the release of contents including mitochondrial DNA (mtDNA) and cytochrome c, which drive caspase-3 activation and apoptosis.5 Caspase-3 activity can also occur locally in neurites and contributes to axonal damage prior to induction of overt neuronal cell death.4 Though the link between mitochondrial damage and caspase-3 activation is well characterized, the myriad mechanisms that amplify local mitochondrial collapse and neurite loss are not clear. Mapping these early molecular mechanisms leading to neuronal dysfunction may have implications for a wide range of neurologic insults. Gasdermins (GSDMs) are a family of pore-forming proteins that have been linked to inflammation and cell death: GSDMA, GSDMB, GSDMC, GSDMD, and GSDME (or DFNA5). GSDMs have pore-forming N-terminal domains that are masked by an autoinhibitory C terminus in the resting state. Proteolytic cleavage in the linker region between the -N and -C domains release the N-terminal fragment of GSDMs to insert and oligomerize in lipid membranes and form pores. The accumulation of GSDM...
pores in the plasma membrane leads to cell swelling and necrosis—a process termed “pyroptosis.”5–9 GSDMD, first identified in macrophages, is activated by caspase 1/11 downstream of inflammasome assembly.8–10 N-GSDMD can also permeabilize mitochondrial membranes leading to the release of cytochrome c, mtDNA, and downstream caspase-3 activation.11–13 The consequences of GSDM activation in a range of cell types has not been well defined. To date, most studies concerning GSDM biology have focused on pyroptotic death in immune or cancer cells.

GSDME, one member of the GSDM family, is expressed in both the brain and spinal cord.14–16 However, its functional role in the nervous system is largely unknown. GSDME activation was first elucidated in cancer cells, where it was shown to be cleaved by caspase-3 to mediate pyroptosis.7 Subsequent work has shown that GSDME can drive cell death in keratinocytes,17 the GI tract,18 kidney,19 mouse retinoblastoma cells,20 and human cancer cells.1,7,14,16

The function of GSDMs in neuronal cell biology and their action in axonal processes has not been studied. Neurons are distinct from other cell types in being both non-dividing and possessing long morphological processes, namely dendrites and axons. Neurons also have unique energetic demands, needing to transport mitochondria over great distances to maintain axons and synapses. In neurodegenerative conditions such as frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), proteins such as TDP-43, SOD1, and C9orf72-associated dipeptide repeat proteins (DRPs) disrupt mitochondrial function and induce loss of axonal processes. These changes are early steps in disease pathogenesis.1,16,21–24

Our study reveals that upon activation, GSDME rapidly distributes to neurite-associated mitochondria to amplify damage, slow mitochondrial trafficking, and promote neurite retraction without the onset of cell death. FTD/ALS-associated proteins induce GSDME activation and localization to axonal mitochondria, driving mitochondrial depolarization and neurite loss. GSDME plays a functional role in driving neurodegeneration in a mouse model of ALS and neurite loss in patient iPSC-motor neuron models. Genetic knockout (KO) or short hairpin RNA (shRNA)-based knockdown of GSDME is neuroprotective both in vitro and in vivo. We identify GSDME as an important modifier of early neuronal mitochondrial damage that may play a role in neurologic disease.

RESULTS

GSDME is expressed in mouse and human neurons
The expression of the GSDM family in the central nervous system and its potential functions in neurons have not yet been well characterized. We mined publicly available single-cell RNA sequencing (RNA-seq) data of mouse cortex and hippocampus sorted for neurons.25,26 The GSDM family members Gsdmd, Gsdma, and Gsdmc were largely absent, whereas Gsdme was expressed in both GABAergic and Glutamatergic neurons (Figure 1A). To further confirm Gsdme expression in the mouse brain, we isolated tissue samples from different brain regions of wild-type (WT) or Gsdme−/− (KO) mice for transcriptional analysis, immunostaining, and immunoblotting. Gsdme mRNA was detected in cortical, cerebellar, midbrain, and spinal cord lysates from WT mice but was absent in Gsdme KO animals (Figure S1A). Anti-GSDME immunostaining of mouse brain showed broad expression in cells with neuronal morphology (Figure 1B). Staining and immunoblotting of mouse brains from KO animals showed negligible immunoreactivity (Figures 1C and S1B).

To assess GSDME enrichment, we performed MACS purification of neurons vs. non-neuronal cells from mouse brain and performed western blot analysis (Figure 1D). We found clear GSDME signals in neuronal fractions that were also positive for neuronal beta-III-tubulin (Tuj1). GSDMD was absent in neurons. However, the non-neuronal fractions (Iba1+, Tuj1−) showed clear expression of GSDME, but not GSDMD (Figure 1D). Consistent with this analysis, transcriptomic data27 from FACS-isolated mouse microglia showed Gsdme expression at baseline but sparse Gsdme detection in these same populations (Figure S1C). Co-staining of cortical and striatal sections showed high colocalization of GSDME with the neuronal marker Tuj1 and not the microglial marker Iba1 (Figure 1E), confirming neuron-specific enrichment of GSDME compared with microglia. Evidence from publicly available human single-cell transcriptomic database demonstrated that GSDME, but not family members GSDMD, GSDMA, or GSDMC, is expressed at baseline in human neurons (Figure S1D).28 Temporal cortices from human brains were stained with anti-GSDME. We found GSDME expression in multiple cortical layers, and these GSDME-positive cells showed a perikaryal staining pattern consistent with cytoplasmic expression in neurons (Figure 1F).

GSDME mediates neuronal damage caused by mitochondrial toxins
Mitochondrial toxins, such as rotenone and 6-hydroxydopamine (6-OHDA), are intrinsic apoptotic stimuli often used to model features of neurodegeneration in vitro and in vivo.29,30 Given that GSDME is expressed at baseline in neurons, we sought to understand if toxin treatment could activate GSDME (Figure 2A). For this study, we utilized several mitochondrial toxins: rotenone and 6-OHDA (complex I inhibitor), antimycin-A (complex III inhibitor), and raptinal (a rapid inducer of mitochondrial damage and

Figure 1. GSDME is expressed in the brain and localizes to neurons at baseline
(A) Single-cell RNA sequencing (Allen Institute for Brain Science) of sorted mouse cortical and hippocampal neurons was mined for expression levels of gasdermins.
(B) Representative IHC images of coronal mouse brain sections stained using an anti-GSDME antibody.
(C) Immunoblot analysis of GSDME expression in several mouse brain regions from adult wild-type and Gsdme knockout mice.
(D) Immunoblot of GSDME and GSDMD expression in neuronal (Tuj1+) and giall (Iba1+) populations from P0 mouse pups.
(E) Representative IHC images of mouse cortex and striatum co-stained with anti-GSDME and either anti-beta-III-tubulin (Tuj1) or anti-Iba1 antibodies. For colocalization, each dot represents a coronal section. Two sections were quantified per mouse (n = 3-5 mice/group).
(F) Representative IHC images of the temporal lobe from two healthy control patients stained with anti-GSDME.
caspase-3). We found that treatment of primary mouse cortical neurons and human SH-SY5Y cells with these mitochondrial toxins led to cleavage of full-length GSDME to the ~30 kDa N-terminal pore-forming fragment by immunoblot analysis (Figures 2B and S2A) and cell death as measured by lactate dehydrogenase (LDH) release and uptake of the membrane impermeant dye propidium iodide (PI) (Figures S1E–S1H). We confirmed specific GSDME cleavage following toxin treatment through immunoblot analysis of WT and GSDME KO SH-SY5Y cells generated using CRISPR-Cas9 targeting (Figure S2A). Thus, these toxins can be used as probes to explore the cell biological functions of GSDME.

We next determined whether loss of Gsdme impacts overt cell death downstream of mitochondrial toxin treatment. Gsdme KO primary mouse neurons showed significantly reduced SYTOX Green or PI uptake compared with WT neurons treated with the toxins, raptinal, rotenone, and antimycin-A (Figures 2C–2G). These results were confirmed in GSDME KO SH-SY5Y cells, which were also protected from PI uptake induced by raptinal, rotenone, 6-OHDA, antimycin-A, and 3-Nitropropionic acid (3-NP) (Figures S2B–S2G). Challenges with ER stressors tunicamycin and thapsigargin also caused a GSDME-dependent PI uptake (Figures S2I–K). Toxin-induced LDH release was also partially rescued in GSDME deficient primary neurons and SH-SY5Y cells compared with controls, although these effects were modest (Figures 2H and S2H).

**GSDME activation in neurons is caspase-dependent**

Given that mitochondrial toxins classically activate caspase-3, which is upstream of GSDME cleavage,6,7,17, we assessed whether GSDME-driven cell death is indeed caspase-dependent in neurons. Treatment with the pan-caspase inhibitor zVAD-FMK blocked raptinal-induced GSDME processing into its N-terminal fragment (Figures S3A and S3B). The incubation of SH-SY5Y and mouse primary cortical neurons with zVAD-FMK phenocopied the effect of GSDME KO, decreasing PI uptake after toxin treatment (Figures S3C–S3F; Video S1). By contrast, treatment with the RIP kinase inhibitors nec-1s and GSK ‘872 did not reduce toxin-induced PI uptake in primary neurons, suggesting that necroptosis does not participate in mitochondrial toxin-driven death (Figures S3G–S3H).

**Activated GSDME localizes to mitochondria**

Neurons have large somas and fine dendritic and axonal processes (neurites). Given this unique morphology, we hypothesized that GSDME may act at specific subcellular compartments to drive cellular dysfunction prior to (or perhaps independent of) cell death. To investigate the intracellular dynamics of GSDME, we transfected mouse cortical neurons with full-length GFP-tagged GSDME (Figure S4A). We validated that GFP-GSDME could be cleaved and maintained pore-forming and pyroptotic ability by transfecting this construct into HEK293T cells, which lack endogenous GSDME expression (Figures S4B and S4C). When transfected cells were treated with raptinal, we observed caspase-3 processing, cleavage of GFP-GSDME, rapid Pi+ uptake, and pyroptotic morphology (Figures S4D–S4F). These changes were not observed in untransfected or GFP-transfected control cells (Figure S4D). Immunoblot time course analysis for GSDME activation revealed that cleavage of both endogenous GSDME or transduced GFP-GSDME were identical and concomitant with caspase-3 cleavage in primary mouse neurons (Figure S4G).

Following raptinal treatment of neurons, GFP-GSDME rapidly formed numerous intracellular puncta in the distal neurites by 45 min and eventually in proximal neurites and cell bodies by 75 min (Figures 2I and 2J). Neuronal GFP-GSDME puncta were predominantly dispersed in the cytosol, in contrast to the human neuroblastoma cell line, that had both plasma membrane and cytosolic enrichment (Figures S5A and S5B). Indeed, in primary GFP-GSDME-expressing neurons treated with raptinal, we observed only a small degree of membrane colocalization 6 to 10 h post-toxin exposure (Figure S5C). The appearance of GSDME puncta in the distal and proximal neurites preceded PI uptake (necrosis) by several hours (Figures 2I and 2J). This data suggests that in neurons, intracellular GSDME localization may occur much earlier than the plasma membrane involvement (and PI uptake) classically described in pyroptosis. To assess whether the neurite-associated puncta represent local sites of activated GSDME, we pre-treated the neurons with zVAD-FMK (caspase inhibitor), at a concentration that blocked GSDME cleavage (Figures S3A and S3B). Early toxin-induced puncta formation was inhibited by zVAD-FMK (Figures S5D and S5E).

Given the ability of GSDME and GSDMD to bind cardiolipin,7 which is enriched on mitochondrial membranes, we tested the hypothesis that intracellular GSDME may be localized to mitochondria along the neurites. Cortical neurons were co-transfected with GFP-GSDME and with mKate-OMP25 to allow visualization of the outer mitochondrial membrane (OMM). We analyzed the fraction of GSDME that colocalized with OMP25 in the distal axons and found raptinal treatment resulted in a 1.5-fold increase in the enrichment of GSDME on axonal and cell body mitochondria (Figures 3A–3C, S6A, and S6B). In addition to raptinal, the mitochondrial toxin, rotenone also caused rapid colocalization of GFP-GSDME with mKate-OMP25+...
mitochondria (Figures S6C–S6E). Immunoblot analysis of fractionated lysates from raptinal-treated SH-SY5Y cells (Figure S6F) and primary cortical neurons revealed a 3-fold enrichment of endogenous cleaved GSDME in mitochondria compared with cytosolic fractions (Figures S6G–S6I). GSDME colocalization with mitochondria was also associated with the release of the inner mitochondrial membrane protein, cytochrome c, into the cytosol (Figures S6I, S6K, and S6L). Previous studies have

Figure 3. Activated GSDME rapidly localizes to neuronal mitochondria following toxin treatment
(A–C) Representative images (A) of a mouse neuron co-transfected with mitochondrial marker mKate-OMP25 and GFP-GSDME imaged before and after raptinal treatment (90 min). (B) The location of mitochondria (red) and GFP-GSDME (green) intensity as measured via line scans along the neurites. (C) The enrichment of green GSDME signal at mitochondria (red) over the background (diffuse cytosolic fluorescence) was quantified from such line scans along neurites. n = 30 neurite segments representing 10 neurons across 3 wells.
(D–F) Representative image (D) of a microfluidic chamber plated with wild-type mouse cortical neurons transfected with GFP-GSDME and mKate-OMP25. The axonal chamber was treated with raptinal for 2.5 h. Images of proximal neurites (left) and a distal axon segment (right) before and after raptinal treatment are shown. Enrichment of GFP-GSDME on mitochondria in (E) proximal and (F) distal chambers were quantified (n = 50 distal and 50 proximal neurite segments taken from three microfluidic chambers).
(G and H) Representative images of mouse neurons transfected with GFP-GSDME and either (G) mKate-OMP25 or (H) Cox8-mCherry. These cells were treated with raptinal, fixed, and imaged using structured illumination microscopy (SIM).
Figure 4. GSDME-deficient neurons are protected from toxin-induced mitochondrial dysfunction

(A) Wild-type and Gsdme knockout primary neurons were stained with TMRM and incubated with rotenone. Images were captured every 15 min and TMRM intensity normalized to DMSO controls.

(B and C) Wild-type and Gsdme knockout primary neurons were transfected with mKate-OMP25 and treated with raptinal. (B) Representative images at 4 h post-raptinal treatment. (C) Mitochondrial density was calculated by counting OMP-25+ objects and dividing by neurite length at 0, 1, 2, and 4 h post-raptinal treatment.

(D–F) Representative transmission electron microscopy images (TEM) of wild-type and Gsdme KO neuronal cultures (D) treated with either DMSO or raptinal for 1 h. (E) Mitochondrial length (dot = 1 mitochondrion) and (F) the percentage of damaged mitochondria (dot = average of 2 wells) were calculated across three independent experiments.
shown that mitochondrial toxins such as rotenone and 6-OHDA cause cardiolipin externalization to the OMM in both primary cortical neurons and SH-SYSY cells. 39,40 shRNA-mediated knockdown of cardiolipin synthase (CLS1) partially decreased GFP-GSDME puncta formation in toxin-treated primary neurons (Figures S7A–S7C). This data suggests that following activation, GSDME localizes to neuronal mitochondria in a partially cardiolipin-dependent manner.

We next asked whether local toxin exposure can induce GSDME activation in axons, without the involvement of the soma. We plated mouse cortical neurons in microfluidic chambers, which allowed for selective treatment of the axonal compartments with toxin (Figure 3D). Raptinal exposure caused GFP-GSDME puncta formation and mitochondrial colocalization in axons without affecting the untreated (soma) compartment of the same cell (Figures 3D–3F). These data suggest that mitochondrial stress leads to the enrichment of GSDME on neuronal mitochondria and can occur locally in axons.

High-resolution structured illumination microscopy (SIM) of toxin-treated neurons transfected with GFP-GSDME revealed robust puncta formation colocalizing with the OMM marker mKate-OMP-25 but not with the matrix marker Cox8-mCherry (Figures 3G and 3H; Videos S2 and S3). These results suggest that toxin treatment targets activated GSDME to mitochondrial membranes.

**GSDME localizes to mitochondria in human iNeurons**

Human iPSC-derived neurons (iNeurons) are postmitotic, electrically active, and thus more representative of human neurons than SH-SYSY. 31 We generated glutamatergic neurons similar to layer 2/3 glutamatergic neurons of the cerebral cortex and investigated GSDME activation in these cells. 32 We found raptinal treatment induces a dose-dependent GSDME cleavage in iNeurons (Figure S8A). The activation of GSDME in iNeurons was blocked in the presence of the caspase inhibitor zVAD-FMK (Figure S8B). iNeurons transfected with GFP-GSDME and mKate-OMP-25 also showed robust mitochondrial colocalization upon toxin treatment, with a large number of mitochondria in the distal neurites (Figures S8C–S8E).

**Gsdme deficiency protects neurons from mitochondrial damage**

We next asked whether GSDME enrichment on neuronal mitochondrial potentiates damage to these organelles. To assess the functional consequences of GSDME mitochondrial colocalization in neurons, we performed tetramethylrhodamine methyl ester (TMRM) staining following toxin treatment. TMRM is a voltage-sensitive mitochondrial dye that loses fluorescence following mitochondrial depolarization. 33 The treatment of WT primary neurons with raptinal, rotenone, or antimycin-A led to dose-dependent TMRM loss. Depolarization was partially rescued in Gsdme KO primary cortical mouse neurons and SH-SYSY (Figures 4A and S9A–S9G). These experiments suggest that downstream of an initial mitochondrial insult (i.e., toxin exposure), GSDME potentiates or accelerates mitochondrial damage. To assess how GSDME impacts mitochondrial membrane integrity we transfected WT and KO cortical neurons with mKate-2-OMP25 and treated with toxin. After 2 and 4 h of treatment (but not by 1 h), Gsdme KO neurons had preserved the OMP-25 signal, suggesting a sparing of the OMM relative to WT cells (Figures 4B and 4C). To directly visualize the structural integrity of mitochondria, we performed transmission electron microscopy (TEM) of WT and Gsdme KO neurons treated for 1 h with raptinal. Analysis of TEM images revealed that raptinal-treated WT mitochondria had less pronounced cristae and increased fragmentation (reduced length) compared with those in Gsdme KO neurons (Figures 4D and 4E). Raptinal-treated WT neurons also displayed a high percentage of mitochondria with damaged inner or outer membranes, whereas Gsdme KO neurons were almost completely rescued (Figure 4F).

Cytochrome-c (cyt-c) release into the cytosol indicates OMM damage. Raptinal treatment caused rapid (<1 h) cyt-c release in WT cortical neurons and SH-SYSY (Figures S6G and S6L). Immunoblots for cyt-c revealed that toxin-treated GSDME KO SH-SYSY displayed a significantly higher mitochondrial to cytosolic Cyt-c ratio relative to WT cells (Figures S6K and S6L), suggesting rescued OMM damage (i.e., Cyt-c release). Consistent with less mitochondrial depolarization and Cyt-c release, GSDME KO SH-SYSY also exhibited reduced caspase-3 activation relative to WT cells (Figures S10A–S10C). 34 Gsdme KO cortical neurons treated with raptinal also had partially reduced caspase-3 activation relative to WT neurons (Figures S10D and S10E); however, the extent of caspase-3 rescue in neurons was less than in SH-SYSY. When taken together, these data indicate that GSDME participates in a positive feedback loop (Figure 2A) to amplify mitochondrial damage and downstream caspase-3 activation in SH-SYSY and primary mouse neurons.

Destruction of axonal mitochondria or disruption of trafficking to distal axons is an early step in many neurodegenerative diseases. 1,3 If GSDME destroys mitochondrial membranes following toxin exposure, we reasoned that fewer mitochondrion would display normal trafficking after toxin treatment. To determine how GSDME impacts mitochondrial trafficking, we transfected primary neurons with mKate-OMP25 and imaged WT and Gsdme KO cells treated with toxins every 5 s. At baseline, we noticed no differences in mitochondrial movement (percent motile) between the two genotypes (Figures 4G–4H). The treatment of WT cultures with raptinal caused a striking arrest of motility at 1 and 2 h post-treatment, as displayed in kymograph analyses (Figure 4G). This arrest was reduced in Gsdme KO neurons, which had more motile mitochondria at these time points (Figure 4H).

(G) Wild-type and Gsdme KO mouse neurons transfected with mKate-OMP25, treated with raptinal, and imaged at high-temporal resolution (1 image/5 s) for 3 min intervals. These intervals were captured at 0, 1, and 2 h post-toxin exposure.

(H) Kymograph analysis was performed to visualize and quantify mitochondrial motility. Percent motile mitochondria were calculated from kymograph analysis of wild-type and Gsdme KO neurons treated with raptinal. Combined data from three independent experiments are shown.
GSDME regulates neurite integrity and mitochondrial potential

Axons require a constant supply of energy to maintain structural integrity and synaptic function. Given that GSDME promotes mitochondrial depolarization and reduces motility following toxin exposure, we hypothesized that Gsdme KO neurons would have reduced degeneration of axonal processes. Following 30 min (raptinal, antimycin-A) or 2 h (rotenone) of treatment with toxins, cells were incubated in fresh media for 8 h and then stained for beta-III-tubulin (Tuj1). WT neurons displayed a dose-dependent increase in the ratio of depolymerized to polymerized beta-III-tubulin (Figures S10F and S10G). Higher levels of depolymerized tubulin indicate microtubule disassembly and axonal degeneration.

Gsdme KO cells demonstrated a striking preservation of axonal processes containing polymerized tubulin (Figures 5A–5D). At these time points, the release of LDH from WT and KO neurons was minimal, indicating that GSDME increases axonal destruction prior to overt cellular necrosis (Figure S10H). The incubation of toxin-treated WT cells with zVAD-FMK also reduced the microtubule depolymerization index, phenocopying the effect of Gsdme KO and agreeing with prior findings (Figure S10I). Collectively, these experiments suggest that the caspase-3/GSDME axis is necessary to disrupt mitochondrial health, motility, and structural integrity in neurites before it causes overt cell death at the soma.

Using microfluidic chambers, we found that GSDME can be locally activated in axons and rapidly colocalizes with mitochondria (Figure 3D). To determine the effect of this local GSDME activation on axonal mitochondrial health, we plated neurons in microfluidic chambers and stained with TMRE (Figures 5E, S10J, and S10K). We examined WT and Gsdme KO axonal compartments treated with toxins for differences in TMRE staining. Raptinal caused a rapid and pronounced loss of TMRE positivity in WT cells (specifically in the treated/axonal chamber), which was partially rescued in Gsdme KO neurons (Figures 5E–5G). Although long-term raptinal exposure leads to total TMRE loss, brief (30 min to 2 h) treatment causes mitochondrial depolarization that is strongly GSDME-dependent (Figure 5G). These data argue that GSDME can be activated locally to destroy mitochondria, in the absence of overt changes at the cell body.

N-GSDME is sufficient to drive mitochondrial damage and neurite loss

The expression of N-terminal GSDME fragment (N-GSDME) in primary mouse neurons was also sufficient to drive mitochondrial colocalization in cell bodies and axons (Figures S11A and S11B). SIM microscopy analysis revealed the overlap of GFP-tagged N-GSDME (GFP-N-GSDME) and OMP-25 in both neurons and SH-SY5Y cells. Only in SH-SY5Y cells did we observe readily apparent GFP-N-GSDME enrichment on the plasma membrane (Figures S11C and S11D). The expression of N-GSDME in primary cortical neurons was also sufficient to cause mitochondrial potential loss (Figures S11E and S11F) and neurite damage as measured by Tuj1 staining (Figures S11G and S11H). Thus, N-GSDME expression phenocopies results obtained following chemical activation of GSDME using toxin treatment.

ALS/FTD-associated proteins induce GSDME activation

Neurodegenerative conditions like ALS and FTD are characterized by early axonopathy that precedes loss of the neuronal soma. Given that GSDME can drive axonal mitochondrial damage prior to cell death, we examined whether neurodegeneration-associated proteins would engage GSDME. To test whether ALS/FTD-associated proteins induces GSDME cleavage and mitochondrial localization in our in vitro system, we focused on the role of TDP-43 and the C9ORF72 hexanucleotide mutation-associated DPR, PR-50. TDP-43 aggregates have been shown to bind mitochondria, leading to complex I inhibition and mitochondrial depolarization. DPRs produced from mutated C9ORF72 have also been shown to impair mitochondrial complex V activity, leading to caspase-3-dependent axon loss.

Mouse cortical neurons were transfected with TDP-43, PR-50, or IRFP control plasmids to model neurodegeneration in a dish. The co-expression of TDP-43 or PR-50 with GFP-GSDME led to increased GFP-GSDME puncta (Figures 6A and 6B). These puncta were enriched on mitochondria along neurites. The treatment of WT cortical neurons with lentiviruses encoding control (GFP), TDP-43 or PR-50 under the control of a synapsin-I promoter, also led to cleavage of endogenous GSDME at 72 h post-transduction, indicating its activation (Figures 6C and S12A).

GSDME mediates TDP43 and PR-50 induced neurite loss

We next asked whether GSDME could contribute to neuronal dysfunction caused by ALS/FTD-associated proteins in mouse neurons. We treated neurons with lentiviruses encoding TDP-43, PR-50, or GFP. WT neurons transduced with TDP-43 and PR-50 exhibited significant mitochondrial depolarization indicated by loss of TMRE staining at 72 and 96 h post-treatment (Figures 6D, 6E, and S12B). Gsdme KO cells had reduced mitochondrial depolarization compared with WT neurons (Figures 6D, 6E, and S12B). These experiments indicate that ALS/FTD-associated proteins can cause GSDME activation, mitochondrial localization, and mitochondrial depolarization that partially relies on GSDME.

Human iNeurons express full-length GSDME at baseline (Figure S8A). To ask whether neurodegeneration-associated proteins could activate GSDME in human neurons, we transfected cortical iNeurons with plasmid encoding TDP-43. Transfected neurons exhibited robust GFP-GSDME puncta that colocalized with mitochondria (Figures S12C and S12D). As in mouse neurons, we found that lentiviral transduction with FLAG-tagged TDP-43 (at multiplicity of infection 4 and 8) led to cleavage of endogenous GSDME relative to GFP-transduced controls (Figure S12E). When taken together, these data suggest that a diverse range of neurotoxic stimuli—mitochondrial toxins, PR-50, TDP-43 can lead to downstream GSDME activation and mitochondrial colocalization in both mouse and human neurons.

Next, we assessed whether these Gsdme KO neurons would have spared neuritic processes. We took two complementary approaches to answer this question. First, we transfected neurons with RFP and either GFP (control) or TDP-43 or PR-50 plasmids. We then measured the neurite area of transfected cells using the RFP expression as a marker. Compared with WT

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Figure 5. GSDME knockout protects against neurite loss and local mitochondrial damage

(A) Representative images of wild-type and Gsdme knockout neurons treated with DMSO, raptinal, rotenone, and antimycin-A and stained for TuJ1, 8 h post-toxin treatment. 

(B–D) Microtubule depolymerization index was calculated for wild-type and KO neurons treated with (B) raptinal, (C) rotenone, or (D) antimycin-A. Violin plots display the median and interquartile ranges for the depolymerization index taken from three independent experiments.

(E) Representative image of a microfluidic chamber plated with wild-type mouse cortical neurons stained with TMRM. The panel represents the chamber prior to the addition of raptinal. The black dashed box indicates an axonal region magnified in (F).

(F) Representative images of axonal segments from wild-type and Gsdme KO neurons before and after (60 min) addition of 5 μM raptinal and stained with TMRM.

(G) Quantification of TMRM intensity relative to baseline (t = 0) from the axonal chambers of plated wild-type and Gsdme KO neurons. Axonal segments from three wild-type and three KO microfluidic chambers were used for analysis (n = 15–17 axons measured across three micorfluidic chambers for each genotype).
neurons, Gsdme KO cells transfected with PR-50 or TDP-43 had preserved neurite area (Figures S12F–S12I). We also used lentiviruses encoding either PR-50 or TDP-43 and measured the resulting microtubule depolymerization index in cortical neurons at 72 h post-transduction. Gsdme KO neurons were protected from neurite loss, with results mirroring those obtained using mitochondrial toxins (Figures 6F–6I). At these time points, there was minimal LDH release from WT and KO cells compared with untransduced controls (Figures 6J and 6K). This indicates that GSDME mediates TDP-43- and PR-50-dependent axonal loss prior to cellular necrosis; thus, GSDME can perform important cellular functions in the absence of cell death.

**GSDME drives neurite loss in patient-derived ALS neurons**

To determine potential relevance to human disease, we mined genome-wide expression profiling data of laser capture microdissection-enriched motor neurons from patients with sporadic ALS (sALS). 32 We found that GSDME was significantly upregulated in neurons from sALS patients relative to age-matched healthy controls, and GSDMD expression was not detected in these cells (Figure S13A).

We next investigated whether GSDME contributes to neuronal dysfunction in human ALS patient iPSC-derived motor neurons. To model human neurodegeneration in *vitro*, we cultured WT iPSC-derived (1016A) and ALS patient iPSC-derived (TDP43 <sup>G298S</sup>) lines that were differentiated into mature MNs. These cells were validated by sequencing, karyotyping and immunostaining for motor neuron marker Islet 1/2 (Figures S13B–S13E). The challenge of these iMNs with a pro-teasome inhibitor (MG132) or ER-stress toxins (thapsigargin and tunicamycin) led to dose-dependent neurite loss and cell death—these effects are exacerbated in the mutant TDP43 <sup>G298S</sup> line (Figures S13F and S14A–S14D). 33

We optimized the knockdown of GSDME in human iPSC-derived motor neurons by testing three GSDME shRNA lentiviruses vs. a scrambled control (Figures S14E and S14F). The shRNA sequences #1 and #3 were validated to efficiently knock down GSDME. TDP43 <sup>G298S</sup> motor neurons (in the presence of a scrambled shRNA) were highly vulnerable to neurite destruction caused by MG132, tunicamycin, and thapsigargin (Figure 7A). The knockdown of GSDME using lentiviral delivery of the two validated shRNAs led to significant sparing of neurite loss in TDP43 <sup>G298S</sup> iMNs (Figure 7). Collectively, these results suggest that GSDME drives pathology downstream of ER stress and proteasome inhibition in vulnerable neurons with a TDP43<sup>G298S</sup> genetic background. Thus, GSDME deficiency can rescue neurite loss in a patient-derived ALS model.

**GSDME impacts survival and motor neuron loss in SOD1<sup>G93A</sup> mice**

We next tested whether GSDME is functionally relevant in an animal model of neurodegeneration. Mice expressing a human SOD1<sup>G93A</sup> transgene (that is linked to ALS) develop progressive motor neuron loss in the ventral spinal cord and paralysis. 34 Both *in vitro* and *in vivo* studies have shown that mutant SOD1 proteins induce mitochondrial dysfunction, impaired ATP production, inefficient calcium buffering, and increased apoptosis of motor neurons. 35,36 To assess the relevance of GSDME in *vivo*, we first probed spinal cord lysates from SOD1<sup>G93A</sup> mice at different time points of disease. We detected GSDME cleavage in the spinal cords of symptomatic SOD1<sup>G93A</sup> mice at postnatal day 140 (P140), which was further increased by the late stage of disease (P160) (Figures 8A, 8B, S15A, and S15B). Cleaved GSDME was not detected in pre-symptomatic P82 animals (Figure S15A). Given this activation pattern, we hypothesized that GSDME may contribute to disease progression in the SOD1<sup>G93A</sup> model. We generated transgenic mice hemizygous for the SOD1<sup>G93A</sup> transgene and were either WT (SOD1<sup>G93A</sup> Gsdme WT) or KO for GSDME (SOD1<sup>G93A</sup> Gsdme KO). By qRT-PCR, we confirmed that experimental animals had similar copy numbers of the SOD1<sup>G93A</sup> transgene irrespective of WT or KO Gsdme genotype (Figure S15C).

Knockout of Gsdme significantly extended survival and delayed disease progression in mice containing the SOD1<sup>G93A</sup> transgene (Figure 8C). Mean survival for SOD1<sup>G93A</sup> Gsdme WT mice (*n* = 31 total) was 159 days (±1.9), whereas that of SOD1<sup>G93A</sup> Gsdme KO mice (*n* = 42 total) was 171 days (±2.5) (Figure 8C). Female SOD1<sup>G93A</sup> Gsdme KO animals received a slightly greater survival benefit (*mean* = 178 days) relative to SOD1<sup>G93A</sup> Gsdme WT mice (*mean* = 160 days) compared with males (Figures 8D, 8E, and S15F). Age to maximum body weight in SOD1<sup>G93A</sup> animals and body weight measurements at all time points were unaffected by the Gsdme genotype (Figures 8F and S15D). This data indicates that disease onset may not be GSDME dependent. However, disease progression—the time
from maximum body weight until euthanasia—was delayed in SOD1\(^{G93A}\) Gsdme KO animals relative to SOD1\(^{G93A}\) Gsdme WT counterparts (Figure 8G). Loss of grip strength was also delayed in SOD1\(^{G93A}\) Gsdme KO compared with SOD1\(^{G93A}\) Gsdme WT mice (Figure 8H). No baseline differences in grip strength were recorded for non-SOD1 transgenic (nTg) Gsdme WT and KO animals (Figure S1SE). These results indicate that GSDME impacts the disease progression phase in this animal model, leading to an overall motor and survival benefit.

Blinded histological counting of Nissl-stained motor neurons in spinal cord ventral horns showed a preservation of neurons in SOD1\(^{G93A}\) Gsdme KO mice at P150 (late-stage) relative to age-matched SOD1\(^{G93A}\) Gsdme WT littermates (Figures 8I and 8J). This is consistent with grip strength and behavioral observations (Figure 8H; Video S4). Microglia and astrocyte activation are hallmarks of disease progression in the SOD1 mouse model of ALS and in human ALS patient spinal cord.\(^{16-50}\) We observed that SOD1\(^{G93A}\) Gsdme KO mice showed the decreased intensity of GFAP+ astrocytes (astroglisis) in the ventral horns compared with SOD1\(^{G93A}\) Gsdme WT animals (Figures S16A and S16B). SOD1\(^{G93A}\) Gsdme KO animals also had decreased microgliosis, as measured by the number of Iba1+ positive cells in the ventral horn and levels of the lysosomal activation marker CD68 (Figures S16C–S16F). Taken together, this data suggests that GSDME is activated in a classic mouse model of ALS and drives pathology—namely neuronal loss and gliosis—that contributes to decreased motor function and survival.

**DISCUSSION**

Mitochondrial dysfunction and axonal loss are important and early hallmarks of neuronal injury. A wide range of upstream insults, such as aggregated proteins, toxins, and age, all converge to induce mitochondrial damage.\(^{1,14}\) Here, we describe GSDME as a key regulator of mitochondrial health and axon loss in neurons downstream of multiple classes of harmful neurotoxic stimuli.\(^{13,51}\) In contrast to prior work describing a role for GSDMs in pyroptotic cell death, we show that GSDME-mediated mitochondrial damage and neurite loss can occur in neurons in the absence of overt cell death.

Thus, we expand the cell biological role for this class of pore-forming proteins, showing that GSDME activation has consequences that are specific to neurons in driving axon loss and distinct from pyroptosis. Prior work has mainly explored GSDME activation in cancer cell lines, focusing on its contribution to cell necrosis.\(^{7,15,52}\) In cancer cells treated with DNA-damaging agents (e.g., UV radiation and etoposide), GSDME colocalizes with mitochondria and mediates cytotoxic c release to amplify caspase-3 processing.\(^{6,52}\) Work in a mouse model of macular degeneration and a murine retinoblastoma cell line has also implicated GSDME in cytochrome-c release and caspase-3 activation in the sensory neuroepithelium.\(^{20}\)

We establish this positive feedback loop in primary neurons and map it locally to axons and neurites. We further show that GSDME-driven mitochondrial damage is critical to amplify downstream neurite loss. That GSDME activity can occur locally in axons, without apparent involvement of the cell body, raises the possibility that GSDME activation could be a key early step in axonopathy.

Neurons may utilize multiple pore-forming molecules to regulate mitochondrial membrane integrity in addition to GSDME—namely, pro-apoptotic BCL-2 family members (e.g., Bax and Bak), and the mitochondrial permeability transition pore (MPTP). Indeed, recent work has shown that GSDMD can act upstream of Bax and VDAC1 channels, promoting their incorporation into mitochondrial membranes. Another study demonstrated that GSDMD mitochondrial pores can promote mitochondrial reactive oxygen species (mtROS) production in macrophages and thereby activate downstream necroptotic machinery.\(^{13}\) This raises the possibility that other pore-forming molecules, such as mixed lineage kinase domain-like pseudokinase (MLKL), could amplify plasma membrane damage in neurons after GSDME-mediated mitochondrial disruption.

 Destruction of axons is an early step in conditions such as neurodegeneration, peripheral neuropathy, and stroke.\(^{16,21,36}\) We believe that our findings using microfluidic compartments are the first to suggest that GSDME activation can be restricted to axons and drive local mitochondrial damage. Thus, our study positions GSDME as a molecule that may work alongside calpains, sterile alpha and TIR motif containing 1 (SARM1), mitogen-activated protein kinase (MAPK)-c-Jun N-terminal kinase (JNK), and other effectors to disrupt axons.\(^{26,34,50,54}\) SARM1 has been shown to regulate the extent of GSDMD-driven pyroptosis in immune cells.\(^{55}\) The MAPK-JNK signaling axis not only promotes SARM1-dependent axonal degeneration in vivo but is also an upstream regulator of GSDME activity in cancer cells.\(^{14,56,57}\) It is of great interest to determine if SARM1, calpains, JNK, and GSDME function in a common pathway leading to axon degeneration.

We found that GSDME is activated by the overexpression of neurodegeneration-associated proteins. TDP-43 has been shown to bind and directly damage mitochondria, leading to the release of mtDNA and cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) activation.\(^{41}\) C9orf72 DPRs directly inhibit mitochondrial complex V, leading to membrane depolarization and neuronal death.\(^{58}\) Motor neurons of patients and mice carrying SOD1 mutations have impaired electron transport chain function, disrupted mitochondrial ATP production and inefficient calcium buffering, and enhanced caspase-3 activation.\(^{23,45,50}\) Thus, multiple neurodegeneration-associated proteins converge as mitochondrial insults and thereby activate the GSDME pathway.

**Figure 7.** GSDME knockdown rescues neurite loss in ALS iPSC-derived motor neurons

(A) Representative TuJ1 staining of control 1016A (wild-type) or TDP43\(^{G93A}\) iPSC-derived motor neurons transduced with either scrambled shRNA or GSDME targeting shRNA and treated with either DMSO (vehicle), tunicamycin, MG132, or thapsigargin (48 h post-toxin treatment).

(B–E) Microtubule depolymerization index was calculated for 1016A (wild-type) and TDP43\(^{G93A}\) motor neurons treated with (B) 0.1% DMSO (vehicle), (C) 1 µM MG132, (D) 0.5 µM of thapsigargin, (E) 5 µM tunicamycin, and either scrambled shRNA or GSDME targeting shRNAs. Violin plots display the median and interquartile ranges for depolymerization index (n = 6 technical replicates).
Figure 8. *Gsdme* knockout rescues SOD1<sup>G93A</sup> pathology in a mouse model of ALS

(A and B) Immunoblots of spinal cord lysates from SOD1<sup>G93A</sup> transgenic mice and wild-type controls (A) at symptomatic phase (P140) and end-stage (P162). (B) The levels of GSDME N-terminal (GAPDH normalized) as well as the ratio of N terminus to full-length GSDME were quantified (n = 3–6 animals) for each time point and genotype.

(C–E) Transgenic mixed-gender mice that were either SOD1<sup>G93A</sup> Gsdme<sup>WT</sup> (n = 31 total) or SOD1<sup>G93A</sup> Gsdme<sup>KO</sup> (n = 38 total) were followed for survival (C). These Kaplan-Meier curves are displayed separately for (D) male SOD1<sup>G93A</sup> Gsdme<sup>WT</sup> (n = 17) and SOD1<sup>G93A</sup> Gsdme<sup>KO</sup> (n = 22) and (E) female SOD1<sup>G93A</sup> Gsdme<sup>WT</sup> (n = 14) and SOD1<sup>G93A</sup> Gsdme<sup>KO</sup> (n = 20) animals.

(F) The age of maximum weight for each mouse was used to as a measure of disease onset (Kaplan-Meier plot).

(G) The time from maximum weight until euthanasia/death was used as a measure of disease progression.

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Previous studies have found that activation of GSDME impacts mouse models of tumor progression, chemotherapy-induced lung damage, cisplatin-induced kidney injury, macular degeneration, and inflammatory bowel disease.\(^7,16,18-26\) We believe our work to be the first report of GSDME’s significance in a classic in vivo (SOD1\(^{G93A}\)) model of motor neuron disease. Mapping how GSDME promotes neuroinflammation—either via neuronal release of pro-inflammatory cytokines, damage-associated molecules, or secreted factors—will be an important basic step in understanding its role in disease pathophysiology.

Our work positions GSDME as an intrinsic executioner of axon degeneration in neurons. When activated by a range of neurotoxic stimuli, GSDME drives the destruction of mitochondria, enhanced caspase-3 activation, neuritic retraction in vitro, and contributes to disease progression in an ALS mouse model. Given its wide expression in the brain, GSDME may function in broad contexts, including in normal development, pathogen infection, and aging, which may warrant future investigation. Small-molecule inhibitors of GSDMD activation have been identified\(^59,60\), although it is unclear whether GSDME is similarly druggable. Future work should determine the feasibility of targeting GSDME expression or function. Ultimately, determining how multiple cell death axes—GSDM, caspase, BCL-2, calpain, and necroptotic machinery—synergeste to enhance neuronal damage may suggest new therapeutic strategies for neurodegenerative disease.

Limitations of the study

In vitro analysis using mouse and iPSC neurons allows for well-controlled pharmacological and genetic manipulation, which is not possible in vivo. However, cultured mouse and human iPSC-derived neurons do not recapitulate the complexity of the nervous system. Although our work shows that Gsdme deficiency is protective in a mouse model of ALS by extending survival and motor neuron survival—we did not directly implicate GSDME-driven mitochondrial damage in disease pathogenesis in vivo—GSDME pores in mitochondrial and plasma membranes could both contribute to disease pathology. Future experiments directly assessing whether SOD1G93A Gsdme deficient animals have increased numbers of intact, polarized mitochondria relative to SOD1G93A counterparts would strengthen our conclusions. Our analysis of human iPSC-derived motor neurons only involves one patient-derived cell line and a normal control—since we do not use isogenic controls, we cannot exclude the possibility that other genetic factors influence our phenotype following GSDME knockdown. Lastly, experiments showing in vivo GSDME cleavage and immunostaining are inherently limited by time resolution—cell death proteins are difficult to detect in vivo due to their transitory nature. The increased sampling of tissues from mouse models and human patients will elucidate how this molecule is regulated during disease.

STAR METHODS

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- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.neuron.2023.02.019.

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(H) Grip strength was tracked for each mouse and normalized by bodyweight.
(I) Representative Nissl-stained images of the lumbar spinal cord from SOD1\(^{G93A}\) Gsdme WT and SOD1\(^{G93A}\) Gsdme KO animals at P150. Magnified regions (white box) delineate a ventral horn area of a spinal cord section.
(J) Quantification of Nissl+ motor neurons per ventral horn of the spinal cord. Each dot represents the average of 6–8 lumbar spinal cord sections from a single mouse.
AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
I.M.C. receives sponsored research support from Abbvie/Allergan Pharmaceuticals and is on the SAB for GSK and LIMM therapeutics. J.L. is a cofounder of Versus Therapeutics. M.E.W. is a consultant for Allergan Pharmaceuticals, Inc. and receives grant support from the NIH and D.V.N. receives grant support from the NIH.

REFERENCES

27. Tabula Muris Consortium; Overall coordination; Logistical coordination; Organ collection and processing; Library preparation and sequencing; Computational data analysis; Cell type annotation; Writing group; Supplemental text writing group; Principal investigators (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. Nature 562, 367–372. https://doi.org/10.1038/s41586-018-0590-4.


regulated by the toll-IL-1R protein SARM. Immunity 50, 1412–1424.e6.


## STAR★METHODS

### KEY RESOURCES TABLE

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**Experimental models: Organisms/strains**

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(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Isaac M Chiu (isaac_chiu@hms.harvard.edu).

Materials availability
Reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability
Enrichment analysis of GSDME on mitochondria, quantification of GSDME puncta and analysis of microtubule depolymerization was done using custom Fiji (ImageJ) macros. The DOI is listed in the key resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
All mouse experimental procedures were performed in compliance with the Harvard Medical School and The Jackson Laboratory Institutional Animal Care and Use Committees. C57BL/6NJ (JAX #005304), C57BL/6N-Gsdme<sup>em1Fsha</sup>/J (JAX #032411), and SOD1<sup>G93A</sup> mice (JAX #002299) were obtained from Jackson labs and bred at Harvard Medical School. Animal experiments were fully approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC). Animals were housed in temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled care facilities at Harvard Medical School on a 12 h light:dark cycle and provided with freely available food and water.

SOD1<sup>G93A</sup> mice
SOD1<sup>G93A</sup> mice (JAX #002299) were originally acquired from Jackson Laboratory (Bar Harbor, ME). The SOD1<sup>G93A</sup> GSDME KO mice were obtained by crossing male mice carrying the SOD1<sup>G93A</sup> transgene with female GSDME KO (JAX #032411) mice for 3 generations. The control SOD1<sup>G93A</sup> GSDME WT mice, were obtained by crossing SOD1<sup>G93A</sup> mice with wild type female mice having a B6NJ background (JAX #005304), also for 3 generations. This breeding strategy controlled for any strain background related phenotypic differences. All offspring were tested for equivalent SOD1 gene copy numbers by qPCR (forward primer: CAGTAACGT AGAGTTTACCTTTGGG; and reverse primer: CACACTAATGCTCGGGAGAAAGA). Mice that had SOD1 signal drop-off of...
more than 30% from control SOD1<sup>G93A</sup> mice were considered as low copy number animals and were excluded from the study. Both male and female mice were used for all experiments. Animals were provided with food and water ad libitum.

**Cell cultures**

**Primary mouse neurons**

Mouse (C57BL/6N or C57BL/6N-Gsdme<sup>em1Fsha/J</sup>) primary cortical neurons were dissociated and cultured using the Worthington Papain Dissociation System (Worthington Biochemical, cat # LK003153). Briefly, post-natal day 0 (P0) mouse cortices were dissected and collected in cold Earle’s Balanced Salts (EBSS). Cortices were then resuspended in 2.5 ml of warmed EBSS with papain (20 units ml<sup>-1</sup>) and Dnase (2000 units ml<sup>-1</sup>). Following a 12 min incubation at 37°C, cortices were triturated 12 times using a 10ml glass pipette, and then 12 times using an 18G needle and syringe to make a single cell suspension. Samples were then passed through a 70um mesh filter to remove debris, and the filtrate was centrifuged (2000xg for 5 min) to pellet cells. Cells were then resuspended in 1.6 ml of suspension media [1.375 ml EBSS, 150 <mu>M albumin-ovomucoid inhibitor (10 mg ml<sup>-1</sup> in EBSS), and 75 <mu>l Dnase (2000 units ml<sup>-1</sup>)]. This solution was layered on top of a 2.5 ml solution of albumin-ovomucoid inhibitor (10 mg ml<sup>-1</sup> in EBSS) to create a continuous density gradient, and the samples were centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and pelleted neurons were collected in warm Neurobasal<sup>TM</sup> Plus medium (Thermo Fisher Scientific) supplemented with 200 mM L-Glutamine with 1% (v/v) penicillin-streptomycin. For plating of primary neurons, dishes were pre-coated with 20 ug/ml poly-L-lysine (Sigma Cat# P2636-25MG) and 6ug/ml laminin (Gibco Cat# 23017-015) in ddH20 for 2h at 37°C. Dishes were washed twice with ddH20 immediately before plating neurons. For GFP-GSDME imaging experiments, cells were plated in 24 well glass bottom dishes (cellVis: P24-1.5H-N) at a density of 3.5 x 10<sup>3</sup> cells per well. For immunoblots, cells were plated at a density of 1 x 10<sup>6</sup> cells per well in 6 well plates (cellVis: P6-1.5H-N). For cell death, TMRM assays and Tuj1 staining, neurons were plated in 96 well glass bottom dishes (Cellvis #P96-1.5H-N) at a concentration of 5 x 10<sup>4</sup> cells/well.

**SH-SY5Y culture**

Wild-type and GSDME<sup>−/−</sup> SH-SY5Y cell lines were a gift from Dr. Judy Lieberman (Boston Children’s Hospital). SH-SY5Y were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 6 mM HEPES, 1.6 mM L-glutamine, 50 <mu>M 2-mercaptoethanol, 100 U/ml Penicillin G and 100 <mu>g/ml streptomycin sulphate. CRISPR-Cas9 knockout of GSDME in SH-SY5Y was performed as previously described. Briefly, GSDME gRNAs (5'-TAAGTTACAGTTCAAGTGC-3' and 5'-TGACAAAAAGAAGAGATCTC-3') were cloned into LentCRISPR-v2 puro vector. The resulting plasmids were transfected into HEK293T with pSPAX2 and pCMV-VSV-G at a 1:1:2 ratio. Supernatants containing lentivirus were collected 2 days later and used to transduce SH-SY5Y at an MOI of 0.3. Two days after transduction, 3 <mu>g/ml (8 <mu>g/ml for CT26) puromycin or 200 <mu>g/ml hygromycin was used to select for positive cells (5d incubation). Cells were then subcloned by limiting dilution in 96-well plates and screened for GSDME expression by immunoblot. LentCRISPR-v2 empty vector was used to generate control cells.

**Human iPSC-derived cortical neuron culture and plating**

NGN2 tetracycline-inducible iPSC neuron lines were a gift from the Tracey Young-Pearse lab and were generated using a lentiviral transduction protocol as previously described. Plates were coated with poly-ornithine and laminin (10 ug/ml poly-ornithine, 5 ug/ml laminin) 24 hours prior to cell plating. Plates were coated with Matrigel basement matrix 2 hours prior to cell plating (8.7 <mu>g/cm<sup>2</sup>). Day 4 cells were thawed from stock and plated at 150,000 cells per well with iN media (Neuralbasal media, 1% Glutamax, 0.3% Dextrose, 0.5% MEM NEAA, 2% B27, 10 ng/mL of each BDNF, GDNF, and CNTF, 5 ug/mL puromycin, 2 ug/mL doxycycline, 10 <mu>M 2-mercaptoethanol immediately before plating neurons. For GFP-GSDME imaging experiments, cells were plated in 24 well glass bottom dishes (cellVis #P96-1.5H-N) at a concentration of 5 x 10<sup>4</sup> cells/well.

**Human iPSC-derived motor neuron culture and plating**

Human induced pluripotent stem cells (iPSCs) were generated as previously described. The TDP43<sup>G98S</sup> line was obtained from a 47-year-old male that was diagnosed with familial ALS (fALS) at age 43, at which point a biopsy was taken. The 1016A wild-type iPSCs were taken from a healthy 20 year old male (Table S3). The cells were cultured on Matrigel-coated (Cat. No. BD354277, VWR) tissue culture plates in StemFlex medium (Cat. No. A3349401, Life Technologies) supplemented with Pen-Strep (Cat. No. 15140163, Life Technologies), maintained at 37°C and 5% CO<sub>2</sub>. Cells were differentiated into motor neurons via chemical-driven, embryoid body (EB)-based protocol as previously described. Briefly, iPSCs were dissociated and grown as suspension cultures in StemFlex medium. After 24 hours, cells were grown in N2B27 media consisting of DMEM/F12 (Cat. No. 12634028, Life Technologies) and Neurobasal media (Cat. No. 21103049, Life Technologies) (1:1), N2 supplement (1% (Cat. No. 17502048, Life Technologies), B27 supplement (2%) (Cat. No. 17504044, Life Technologies), Glutamax (1%) (Cat. No. 35050079, Life Technologies), β-mercaptoethanol (0.1%) (Cat. No. 21985023, Life Technologies), ascorbic acid (20 <mu>M) (Cat. No. A4403, Sigma Aldrich), and Pen-Strep (1%), with additional supplements based on day of differentiation. On days 0 and 1, cells were fed with N2B27 media supplemented with SB 431542 (10 <mu>M) (Cat. No. 1614, R&D Systems), LDN 193189 (100 nm) (Cat. No. 04-0074-02, ReproCELL), and CHIR 99021 (3 <mu>M) (Cat. No. 04-0004-10, ReproCELL). On day 2, cells were fed with N2B27 media supplemented with SB 431542 (10 <mu>M), LDN 193189 (100 nm), CHIR 99021 (3 <mu>M), retinoic acid (1 <mu>M) (Cat. No. R2625, Sigma Aldrich), and smoothened agonist (SAG) (1 <mu>M) (DNSK International). On day 4, media was replaced with day 2 media. On day 5, media was replaced with N2B27 media with retinoic acid (1 <mu>M) and SAG (1 <mu>M). On day 7, cells were fed with N2B27 media with RA (1 <mu>M), SAG (1 <mu>M), and BDNF (20 ng/ml) (Cat. No. 248-BD, R&D Systems). On day 9, media was replaced with N2B27 supplemented with RA (1 <mu>M), SAG (1 <mu>M), BDNF (20 mg/mL) and gamma secretase inhibitor (DAPT, 10 <mu>M) (Cat. No. 2634, R&D Systems). On day 11, cells were fed with N2B27 with RA (1 <mu>M), SAG (1 <mu>M), DAPT (10 <mu>M), BDNF (20 ng/ml), and GDNF (20 ng/mL) (Cat. No. 212-GD, R&D Systems). On day
13, media was replaced with day 11 media. On day 15, EBs formed were dissociated with 0.25% Trypsin-EDTA (Cat. No. 25200114, Life Technologies, counted, and centrifuged at 400 x g for 5 minutes. Pelleted cells were resuspended in complete MN media consisting of Neurobasal media, N2 supplement (1%), B27 supplement (2%), Pen-Strep (1%), Glutamax (1%), nonessential amino acids (1%) (Cat. No. 11-140-050, Gibco), jME (0.1%), ascorbic acid (20 μM), BDNF (20 ng/mL), GDNF (20 ng/mL), CNTF (20 ng/mL) (Cat. No. 130-096-336, Miltenyi), and UFDU (10 μM) (Cat. Nos. U3750/ F0503, Sigma Aldrich). Plated cells were cultured for an additional 7 days prior to experimentation, with half media changes every 3-4 days. The human pluripotent cell lines were reviewed by the Harvard Committee on the Use of Human Subjects and determined to not be human subjects research.

**METHOD DETAILS**

**SOD1 G93A behavioral analysis**

**Weight Measurement**

Weights of all mice carrying the SOD1\textsuperscript{G93A} transgene were measured biweekly from week 7 to week 21. Disease onset was defined as the day of peak weight while disease progression was defined as the number of days between disease onset to euthanasia.

**Grip strength**

Grip strengths of the SOD1\textsuperscript{G93A} transgenic mice were measured weekly, starting at week 7 and ending at week 21. A grip strength machine (BIOSEB # bio-GS3) with a mesh grid attachment was used to obtain combined grip strengths of all four limbs. For each measurement, the mouse was held at the base of the tail and placed on the mesh grid. A total of 5 pulls were performed per mouse with approximately 5-10 second breaks between each pull. The top three pulls per time point were averaged and used for data analysis. 12 or more mice per genotype, per sex, were used for weights and grip strength analysis.

**Survival**

Each mouse was monitored daily after symptom onset. Mice that were unable to rear were given hydrogels and wetted food pellets at the bottom of the cage. Euthanasia time points for each mouse were determined by the inability to successfully right itself when flipped on either of its sides within 30s.

**Brain and spinal cord tissue lysis**

For analysis of mouse CNS tissue, animals were anesthetized with Avertin solution (500 mg kg\textsuperscript{-1}, MilliporeSigma) and perfused with 20 ml of cold PBS before harvest. Brains and spinal cords were dissected in dish of cold PBS and stored at -80°C prior to tissue homogenization/lysis. Lysis buffer was prepared on ice as follows: 10 ml T-per buffer (Thermo #78510), 1 tablet protease inhibitor (Sigma #11836153001/Roche), 100ul HALT protease inhibitor (Thermo #87786), 100ul 0.5M EDTA (Thermo #87786), 1 tablet Phostop\textsuperscript{TM}, phosphatase inhibitor tablets (Sigma #0490685001/Roche). Tissue lysis was performed as previously described.\textsuperscript{63} Briefly, dissected and frozen tissue was resuspended in cold lysis buffer in a 2mL Eppendorf tube. One autoclaved metal bead (BB) was placed in each tube containing sample and lysed in a bead beater (Qiagen TissueLyser II) at a setting of 25 Hz for 5 minutes. Homogenized tissue samples were then incubated on a rotating tube rack at 4°C for 30 min. Following incubation samples were clarified by spinning at 16000 x g for 15mins at 4°C. Supernatants (lysate) were then taken and stored at -80°C prior to analysis. For immunoblot processing, sample lysates were diluted in Bolt 4X loading buffer (Thermo #B0007) containing 1X Bolt Sample Reducing Agent (Thermo #B0009) and beta-mercaptoethanol. Samples were then boiled for 10 min at 90 C, spun down and stored at -20°C.

**Drugs and treatments**

Raptinal (cat# SML1745), rotenone (cat# R8875), antymicina-A (cat# A8674) and 3-Nitroproponic acid (cat# N5636) were purchased from Sigma and resuspended in DMSO to make stock solutions. For raptinal treatments, cells were exposed to final concentrations ranging from 1-10μM for 1h, and then incubated with fresh media and assessed at later timepoints. For antymicina-A treatments, cells were exposed to final concentrations ranging from 5-20 μM for 30 min and then incubated with fresh media. For rotenone and 3-NP treatments, cells were exposed to working concentrations ranging from 10-30μM (Rotenone) and 1-3 mM (3-NP) for 2h and then incubated with fresh media. Z-VAD-FMK (R&D cat# FMK001) was resuspended in DMSO and used at a final concentration of 20 μM. For experiments using toxins and z-VAD, neurons were pretreated with 20μM z-VAD for 30 min, and then treated with a combination of zVAD (20μM) and toxins for the indicated timepoints.

**Primary neuron transfection**

Primary neurons were transfected at DIV 6-7 and analyzed at DIV9-10 (2-3 days following transfection). Transfections were performed using lipofectamine 2000 reagent (Thermo Scientific cat# 11668027) as previously described.\textsuperscript{67} Briefly DNA and lipofectamine mixes were made in plain neurobasal media (NBM, without PSG and B-27) with 0.25-1μg of DNA and 0.75-3μL of lipofectamine reagent (1:3 DNA/lipofectamine ratio). The DNA and lipofectamine mixes were gently vortexed (30s) and incubated at RT for 15 min. Prior to addition of the transfection mix, conditioned media was removed from neuronal plates and saved. Neurons were then washed 3 times with warmed plain NBM, and DNA/lipofectamine mix (100uL/well for a 24 well plate) was added to each well and incubated for 1h at 37°C. Following incubation, each transfected well was washed 3 times with fresh plain NBM and the conditioned media was added back to each well. Neurons were then incubated for 2-3 days at 37°C prior to imaging and analysis.
Neurons were transduced overnight with lentiviruses encoding control (eGFP) or PR-50 and TDP-43 proteins as described previously. Briefly, primary neurons plated in 96-well plates at a density of 50-75,000 cells/well were grown for 72 hours. The neurons were then treated with titered viruses at MOIs of 2 or 3. Transduction volume was 40uL/well. Following overnight incubation with virus, neurons were washed 3x with warmed complete NBM and followed for TMRM staining, immunoblot analysis or Tuj1+ staining as described below.

Live cell imaging of neurons

For live-cell imaging of cortical neurons, neuronal cultures grown on glass bottom dishes at a density of 175,000 cells/cm² were imaged on a DMi8 Zeiss microscope. The microscope was equipped with an environmental chamber that was supplied with humidified 5% CO2 and maintained at 37°C. Images were acquired with an Andor Zyla sCMOS camera via a 20x Plan Apo objective. To monitor GSDME enrichment on mitochondria, images from neurons co-transfected GFP-GSDME and mKate2-OMP25 (referred to as mito-RFP) were captured every 15 minutes following treatment with toxins (raptinal and rotenone). To monitor mitochondrial motility, each wall of neurons (350,000 cells/well) were sparsely transfected with mKate2-OMP25 (referred to as mito-RFP) and cytosolic GFP. Two days following transfection, the neurons were treated and imaged. Images of whole neurons (all visible neurites) were captured every 6s. Kymographs were generated from 3- to 5-min time-lapse movies and analyzed with a custom-written ImageJ macro for percent motility mitochondrial density, and length. High resolution 3-5 min timelapse movies were taken at 30 min, 1h and 2h post-raptinal treatment.

Immunoblotting

Cells were lysed on ice in 1x RIPA buffer (EMD Milipore cat# 20-188) supplemented with 0.5mM EDTA, 1X Halt Protease inhibitor cocktail (Thermo Scientific cat# 87786) and 1 Complete Mini protease inhibitor tablet (Sigma cat# 11836153001). Following a 15 min incubation in lysis buffer, cells were centrifuged at 18,000 x g for 15 min at 4°C. Pellets were discarded and supernatants were diluted with 4X Laemml buffer (to 1X final concentration) supplemented with 10X Bolt sample reducing agent (cat# B0009). Samples were then incubated on 90°C heat block for 10 minutes and run on Bolt 4-12% Bis-Tris-Plus gels (Thermo Scientific cat# NW04125BOX). Gels were transferred to nitrocellulose iBlot 2 membranes (Fisher Scientific cat# IB23001), blocked with 5% Pierce Clear Milk Blocking Buffer (Thermo Scientific cat# 37587) for 30 minutes, washed 3x with TBST (TBS, 0.05% Tween-20), and incubated overnight in blocking buffer containing primary antibody at 4°C. Following antibody incubation, blots were washed 3x with TBST for 10 min, incubated with secondary antibody for 1h at RT, followed by three additional 10 min washes with TBST. GSDME and caspase-3 immunoblots were developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific cat# 34080) on a ChemiDoc MP or Azure 300 chemiluminescent imaging system. GAPDH immunoblots were incubated with IR-fluorophore conjugated secondary antibodies (LI-COR Biosciences cat# 926-32213) and developed and imaged on a Li-COR imaging system.

Cell death assessment (LDH and PI assays)

Neurons were plated at 7.5 x 10⁴ cells/well of a tissue culture-treated black 96 well plate coated with laminin and poly-L-lysine with optically clear flat wells 7d prior to experimentation. Propidium iodide or SYTOX green were diluted in complete NBM to a final concentration of 1.5uM or 1uM respectively and added to neurons during treatment with toxin incubation as described above. Positive control wells were lysed by the addition of 5 ul of 10x lysis buffer (10% v/v Triton X-100) 45 min prior to the desired endpoint for analysis. Propidium iodide uptake was analyzed using the IncuCyte S3 Live cell analysis imaging system and associated software (Sartorius V2018B). To generate time course curves, images were acquired with the IncuCyte ZOOM Plan fluor 20X/0.45 objective (Sartorius cat#4446) every 30 min following PI staining and toxin treatment. PI positive neurons were scored as cells with a threshold signal greater than 2 red calibrated units (RCU) above background, using a Top-hat background subtraction method. Percent maximal uptake was calculated by normalizing experimental wells by the average PI uptake of the triton (positive control) treated wells. For LDH release assays, 50 ul of supernatants from the PI uptake experiments described above were transferred to a clear flat-bottom 96-well plate and assessed using the CytoTox LDH release assay from Promega (cat# G1780) in accordance with the manufacturer’s protocol. Absorbance values (490nm) were obtained on a Bio-Tek Synergy HTX plate reader and analyzed using Gen5 software.

TMRM assays

Image-IT TMRM (Thermo Scientific cat# I34361) reagent was used at a concentration of 100 nM (1:1000 dilution in NBM). Primary neurons were incubated with TMRM solution for 1h at 37°C, and then washed with fresh NBM. Cells were treated with toxins or lentiviruses as described above and imaged every 30 min using the IncuCyte S3 Live Cell Imaging system. Image acquisition and data analysis were performed using the IncuCyte S3 Software (Sartorius V2018B) TMRM positive neurons were scored as cells with a threshold signal greater than 2.5 red calibrated units (RCU) above background, using a Top-hat background subtraction method. Total TMRM intensity was calculated for each well and normalized to DMSO control or vehicle conditions.

Beta-III-Tubulin (Tuj1) immunocytochemistry

Primary cortical neurons grown on laminin and poly-L-lysine-coated 96-well glass bottom plates were treated with mitochondrial toxins or TDP-43/PR-50 encoding lentiviruses and stained using standard immunocytochemistry. Briefly, cells were fixed with 4%
paraformaldehyde and 15% sucrose in PBS for 1h at RT, rinsed 3 times with PBS, and permeabilized using 0.3% Triton-X-100 in PBS for 1h at RT. Following permeabilization, cells were blocked with 1% BSA in PBS for 1h at RT. Fluorescently conjugated 647-Tuj1 antibody (Biolegend chtag# 801210) was diluted 1:300 in 1% BSA + 0.3% Triton-X-100 in PBS. Cells were then incubated overnight in Tuj1 antibody solution at 4C, and then rinsed 3x with PBS. Cells were then mounted with Prolong Diamond antifade mounting media (Cell Signaling cat# 8961S) and imaged using a Leica Thunder microscope with Andor Zyla sCMOS camera via a 20x Plan Apo objective.

**RNA isolation and qPCR**
For analysis of mouse brain tissue, animals were anesthetized with Avertin solution (500 mg/kg, Millipore Sigma) and perfused with 20 mL of cold PBS. Mouse brains were dissected on ice in cold PBS. RNA was then isolated using the RNasea mini kit (Qiagen). Per the supplier, RNA was isolated by a modified guanidium thiocyanate method and integrity and purity was confirmed using an Agilent 2100 Bioanalyzer. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (ThermoFisher Scientific) on a StepOnePlus RT PCR system (Applied Biosystems) or a LightCycler 96 (Roche). Expression relative to Gapdh was calculated using the comparative C_T method.

**Microfluidic chamber assays**
For fluidic isolation of local axon segments, neuronal cultures were established in microfluidic devices (RD450 or XC450 from Xona Microfluidics). The silicone microfluidic devices (RD450) were attached onto a glass coverslip (pre-coated with poly-L-Lysine and Laminin) before use. The microfluidic devices were loaded with freshly dissociated neurons at a density of 500,000 cells per device. Following the initial loading of the neurons into one chamber of each device, they were incubated at 37°C for 30 min to allow the cells to adhere. Afterwards, both chambers of the microfluidic devices were filled with fresh media. The neurons were grown for 9 days in the microfluidic chambers prior to imaging, to allow for sufficient number of axons to grow through the 450µm chamber. During this establishment phase of the neuronal cultures, the growth media in the chamber housing the cell bodies was exchanged with 50% fresh media every 3 days. Neurons grown in microfluidic devices were transected with lipofectamine 2000 in the same way as described for other cultures. The transfection was carried out in the chamber containing the cell bodies. Transfected neurons were imaged two days following transfection.

**Mouse brain and spinal cord immunohistochemistry**
Adult mice were perfused with 20mL of cold PBS and 20mL of 4% PFA at 4C, paraffin embedded and processed for IHC as described. Briefly paraffin embedded sections were dehydrated in successive washes with xylene and ethanol. Sections were then washed in water and boiled in 1X EDTA unmasking solution (Cell Signaling cat#14746). Tissue sections were then incubated in a 3% H2O2 solution for 10 min, washed in TBST-Tween20 and blocked for 1h at room temperature with TBST/5% Normal Goat Serum (Cell Signaling Cat#5429). Following blocking, tissue sections were incubated in dilute primary antibody solutions of antibodies against Tuj1 (1:200 dilution), GFAP (1:100 dilution), Iba1 (1:200 dilution), GSDME (1:300 dilution) or CD68 (1:200 dilution) overnight at 4C. The next day, sections were washed with 1X TBST and incubated for 30min at room temperature in SignalStain® Boost IHC detection Reagent (HRP rabbit, #8114 or HRP mouse, #8125) specific to the species of the primary antibody. Slides were washed with TBST prior to Tyramide Signal Amplification (TSA). Fluorescein conjugated TSA reagent (Akyoa Biosciences, NEL74SO01KT) or Cy3 reagent (Akyoa Biosciences, NEL744001KT) were diluted as per manufacturer’s instructions. Slides were incubated with TSA reagent for 10min at room temperature (protected from light). Slides were then washed three times with 1X TBST, counterstained with DAPI and mounted in Prolong gold antifade mounting medium (Cell Signaling cat# 8961S) and imaged using a Leica Thunder microscope with Andor Zyla sCMOS camera via a 20x Plan Apo objective. For serial staining of sections (dual IHC) a stripping step was performed by boiling slides for 10 min in a 10mM sodium citrate buffer (Cell Signaling cat# 14746). Following stripping, slides were incubated in primary antibody solution, washed, incubated with SignalStain Boost IHC secondary detection reagent and subject to TSA amplification as described above. For Nissl staining of spinal cords, paraffin embedded sections were deparaffinized in xylene 3x10 min. Sections were then hydrated in 100% ethanol 2x5 min, 95% ethanol 1x3 min, 70% ethanol 1x3 min and then rinsed in distilled water. Sections were then stained with Neurotrace Green solution diluted 1:400 in PBS for 1x15 min, rinsed with PBS 0.1% Triton for 1x5 min, washed in PBS for 2x5 min and then mounted in Prolong Gold media for imaging.

**Human brain immunohistochemistry**
Postmortem temporal cortical tissues from FTD/ALS patients with C9orf72 repeat expansions and TDP-43 pathology, patients with Lewy Body Dementia (LBD), and healthy controls were obtained from the UPenn Center for Neurodegenerative Disease Research Biobank. Information on human patients is provided in Table S1. Written informed consent was obtained from legal next of kin. Human tissues were processed and stained as described. Briefly, slides were deparaffinized in xylene, and dehydrated in successive ETOH washes. After a brief wash in dH20, slides were then incubated for 30 min in a 70% MeOH and 30% H2O2 solution, and then washed in tap water. Microwave antigen retrieval was performed for 20 min in a citrate buffer (Vector Labs #H-3300). Slides were then cooled to room temperature, rinsed in TBS (0.1 M Tris Buffer) and subject to blocking solution (TBS/2%FBS/3% PFA at 4C, paraffin embedded and processed for IHC as described. Briefly paraffin embedded sections were dehydrated in successive washes with xylene and ethanol. Sections were then washed in water and boiled in 1X EDTA unmasking solution (Cell Signaling cat#14746). Tissue sections were then incubated in a 3% H2O2 solution for 10 min, washed in TBST-Tween20 and blocked for 1h at room temperature with TBST/5% Normal Goat Serum (Cell Signaling Cat#5429). Following blocking, tissue sections were incubated in dilute primary antibody solutions of antibodies against Tuj1 (1:200 dilution), GFAP (1:100 dilution), Iba1 (1:200 dilution), GSDME (1:300 dilution) or CD68 (1:200 dilution) overnight at 4C. The next day, sections were washed with 1X TBST and incubated for 30min at room temperature in SignalStain® Boost IHC detection Reagent (HRP rabbit, #8114 or HRP mouse, #8125) specific to the species of the primary antibody. Slides were washed with TBST prior to Tyramide Signal Amplification (TSA). Fluorescein conjugated TSA reagent (Akyoa Biosciences, NEL74SO01KT) or Cy3 reagent (Akyoa Biosciences, NEL744001KT) were diluted as per manufacturer’s instructions. Slides were incubated with TSA reagent for 10min at room temperature (protected from light). Slides were then washed three times with 1X TBST, counterstained with DAPI and mounted in Prolong gold antifade mounting medium (Cell Signaling cat# 8961S) and imaged using a Leica Thunder microscope with Andor Zyla sCMOS camera via a 20x Plan Apo objective. For serial staining of sections (dual IHC) a stripping step was performed by boiling slides for 10 min in a 10mM sodium citrate buffer (Cell Signaling cat# 14746). Following stripping, slides were incubated in primary antibody solution, washed, incubated with SignalStain Boost IHC secondary detection reagent and subject to TSA amplification as described above. For Nissl staining of spinal cords, paraffin embedded sections were deparaffinized in xylene 3x10 min. Sections were then hydrated in 100% ethanol 2x5 min, 95% ethanol 1x3 min, 70% ethanol 1x3 min and then rinsed in distilled water. Sections were then stained with Neurotrace Green solution diluted 1:400 in PBS for 1x15 min, rinsed with PBS 0.1% Triton for 1x5 min, washed in PBS for 2x5 min and then mounted in Prolong Gold media for imaging.
BSA) for 5 min at room temperature. Following blocking, anti-Human GSDME antibody (abcam 230482, rabbit-anti-human) was diluted 1:100 in blocking solution. Sections were incubated in GSDME primary antibody overnight at 4°C in a humidified chamber. The next day, slides were washed with TBS, blocked for an additional 5 minutes and exposed to vector biotinylated anti-Rb IgG secondary antibody (Vector #BA-1000) at a dilution of 1:1000 for 1h at room temperature. Slides were then washed in TBS and Vector ABC solution (Vector # PK-4001) was applied to each slide for 1h at RT. Slides were then exposed to Vector IMPAACT DAB solution at room temperature. Following development, samples were washed in dH2O, counterstained using hematoxylin (Cell signaling #14166), rinsed in tap water, dehydrated in successive ETOH and xylene rinses and mounted for imaging.

**Transmission Electron microscopy**

Primary mouse neurons were cultured for 7d (as described above). WT and GSDME KO cells were then treated with 5uM raptinal for 1h, prior to processing for electron microscopy. Cells were fixed for at least 2 hours at RT in a 2.5% Glutaraldehyde 1.25% Paraformaldehyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) solution. Following fixation, neurons were washed in 0.1M cacodylate buffer and postfixed with 1% Osmium tetroxide (OsO4)/1.5% Potassium ferrocyanide (KFeCN6) for 1 hour, washed twice with water, once with Maleate buffer (MB), and then incubated in 1% uranyl acetate in MB for 1h followed by 2 water washes and subsequent alcohol dehydration in the following gradations: 10min each; 50%, 70%, 90%, 2x10min 100%. After dehydration propyleneoxide was added to the dish and the cells were lifted off using a transfer pipet, pelleted and infiltrated ON in a 1:1 mixture of propyleneoxide and TAAB Epon (TAAB Laboratories Equipment Ltd, https://taab.co.uk). The following day the samples were embedded in TAAB Epon and polymerized at 60 degrees C for 48 hrs. Ultrathin sections (about 60nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG Spirit BioTWIN and images were recorded with an AMT 2k CCD camera. For analysis, the average mitochondrial length and number of mitochondria per cell was measured using ImageJ. These parameters were compared/normalized to values from WT and KO neurons treated with DMSO.

**Data mining of sc-RNA-seq databases**

Single cell transcriptomic data from mouse cortical and hippocampal neurons was obtained from the Allen Brain atlas: https://celltypes.brain-map.org/maseq/mouse_ctx-hpf_10x?selectedVisualization=Heatmap&colorByFeature=Cell_Type&colorByFeatureValue=Gad1. Human neuronal single cell data from cortex and hippocampus was obtained and mined from the Allen Brain atlas: https://portal.brain-map.org/atlases-and-data/maseq-human-m1-10x. RNA-seq data of FACS-isolated microglia was obtained from the Tabula Muris database co-hosted by the Chan-Zuckerbergh Biohub and Broad Institute: https://tabula-muris.ds.czbiohub.org/

**Stressing of human non-disease and ALS patient-specific induced pluripotent stem cell derived motor neurons.**

For GSDME knockdown experiments, MNs were plated at 100,000 live cells/well in a 96-well plates pre-coated with poly-L-ornithine (Cat. No. P3655, Sigma Aldrich), poly-D-lysine (Cat. No. A3890401, Life Technologies), fibronectin (Cat. No. 47743-654, VWR), and subsequently antibiotic at (Vector #BA-1000) at a dilution of 1:1000 for 1h at room temperature. Slides were then washed in TBS and Vector ABC solution (Vector # PK-4001) was applied to each slide for 1h at RT. Slides were then exposed to Vector IMPAACT DAB solution at room temperature. Following development, samples were washed in dH2O, counterstained using hematoxylin (Cell signaling #14166), rinsed in tap water, dehydrated in successive ETOH and xylene rinses and mounted for imaging.

**GFP-GSDME image analysis**

**Colocalization analysis of GSDME and mitochondria along neurites**

For analyzing the enrichment of GSDME on mitochondria along neurites, the neurites were tracked manually. The mitochondrial intensity and the GSDME intensity were then measured along the neurites. The mitochondrial intensity was then converted to 1-D masks (by thresholding according to local contrast) while the GSDME intensity was normalized to the average intensity along the neurite. The enrichment of GSDME was then calculated as the ratio of mean GSDME signal within the mitochondrial mask to the mean GSDME signal outside the mitochondrial masks.

**Colocalization analysis of GSDME and mitochondria in cell bodies and SHSY5Y cells**

To analyze the enrichment of GSDME on mitochondria in cell bodies and SHSY5Y cells, the cell outline was drawn manually. The mitochondrial intensity within the cell outline was then used to create masks (by thresholding according to local contrast) and the GSDME intensity was normalized to the average intensity of the whole cell. As done in the case of neurites, enrichment of GSDME on mitochondria was reported as the ratio of mean GSDME signal within the mitochondrial mask to the mean GSDME signal outside the mitochondrial masks.

**Quantification of GSDME puncta**

To quantify the density of puncta along neurites, the neurites were manually traced. The intensity of GSDME was then measured along the neurites and normalized to the average intensity along the whole neurite. To detect GSDME puncta, the GSDME signal was then converted to a mask using a local thresholding window of 20px. The density of GSDME puncta in the cell bodies and SHSY5Y cells was quantified using a similar approach. In this case, the cells were traced manually, following which the GSDME puncta were detected by local thresholding.
Analysis of Beta-III-Tubulin (Tuj1) immunocytochemistry

The microtubule (MT) depolymerization index was measured using a custom macro adapted from Maor-Nof et al.35 Briefly, the polymerized microtubules were distinguished from depolymerized ones using brightness and circularity. Polymerized microtubules are less bright and less circular than the aggregated puncta formed by depolymerized microtubules. In this macro, a low threshold is set at first, to detect and mask all TUJ1 positive objects (including polymerized and depolymerized MTs). In the second step, a high threshold is set, which can distinguish only the bright spots formed by depolymerized MTs. Once detected and masked, the pixels marking depolymerized MTs are removed from the set of pixels (mask) marking all microtubules, as detected in the first step, to get the mask of pixels containing polymerized microtubules only. In the final step, the masks of polymerized and depolymerized microtubules are then refined using a circularity threshold. Any tubular objects detected in the mask of depolymerized microtubules or any solid round objects detected in the mask of polymerized microtubules are removed. The MT depolymerization index is then measured as the following ratio:

$$MT \text{ depolymerization index} = \frac{\text{area of depolymerized microtubules}}{\text{(area of depolymerized microtubules)} + \text{area of polymerized microtubules}}$$

All thresholds used for MT depolymerization analysis were determined prior to analyzing full datasets, using negative and positive controls.

Quantification of mitochondrial motility in neurites

To quantify mitochondrial movement in neurites, images of fluorescently marked mitochondria were captured every 6s. Kymographs were generated from 3 to 5-min time-lapse movies and analyzed with Kymolyzer, a custom-written ImageJ macro. Each data point in the percent motility quantifications represent the average percent time spent in motion by all mitochondria in a neurite segment.

QUANTIFICATION AND STATISTICAL ANALYSIS

For cell culture experiments, statistical analysis was performed with a two-tailed unpaired method (Student’s t-test) for two independent groups, two-tailed pairwise t-tests to assess the effect of toxin or agent on the same sample before and after treatment, and one-way ANOVA for multiple groups with a single variance, all calculated with GraphPad Prism 8. For experiments treating WT and GSDME KO cells with several different toxins or agents, two-way ANOVA (row factor = treatment, column factor = genotype) followed by multiple comparisons was done to compare each group (adj p-values were calculated by the Tukey method). Data shown throughout represents an average of at least 2-3 independent experiments + SEM. Each independent experiment had at least > 3 technical replicates (e.g. wells in culture plate) per condition. Significant statistical figure outcomes must obey a p-value upper bound less than 0.05 and corrected using the Tukey method. Precise statistical details (number of biological replicates, exact comparisons and tests) are detailed in the relevant figure legends. For datasets from the SOD1 mouse model in Figure 7, Kaplan-Meier curves were constructed, and survival analysis was performed. Groups were compared for statistical differences using the log-rank test (Mantel-Cox). For datasets 7G and 7J, student’s t-tests were performed for comparisons between genotypes, and p-values were adjusted using the Tukey method. For dataset 7H, a two-way ANOVA (row factor = time, column factor = genotype) was performed, followed by multiple comparisons for each group (p-values adjusted by the Tukey method are shown).
Supplemental information

Gasdermin-E mediates mitochondrial
damage in axons and neurodegeneration

Supplemental Figures and Tables:

Figure S1
Figure S1: GSDME is expressed in mouse and human neurons (related to Figure 1 & 2)

(A) Gsdme RT-qPCR of several mouse brain regions from WT (n=3) and Gsdme knockout (n=2) mice
(B) Representative IHC images of cortical mouse brain sections from WT and Gsdme knockout animals stained with anti-GSDME and counterstained with DAPI.
(C) FACS-isolated brain myeloid cell transcriptomic data from the publicly available Tabula Muris database was mined for Gsdme and Gsdmd expression. TSNE plots showing cell types (left most plot) and expression of gasdermin genes (right) were generated using the CZI biohub tool (Available from: https://tabula-muris.ds.czbiohub.org/)
(D) Publicly available human cortical single-cell RNA-seq (10X Genomics) data was mined for gasdermin family expression levels. TSNE plots showing cell types (left most plot) and gasdermin genes were generated using the Allen Brain Atlas Transcriptomics Explorer tool (Allen Institute for Brain Science. Available from: human.brain-map.org)
(E) SH-SY5Y cells were incubated in propidium iodide (PI) containing media and treated with 5uM raptinal, 20uM rotenone or 30uM of 6-OHDA. Images were taken every 3h and quantified for PI uptake.
(F) Primary neurons were incubated in propidium iodide (PI) containing media and treated with 5uM raptinal, 20uM rotenone and subsequently imaged to quantify PI uptake.
(G) SH-SY5Y were treated with 5uM raptinal, 20uM rotenone or 30uM of 6-OHDA and assessed for LDH release at 24h post-treatment.
(H) Primary neurons were incubated in media containing multiple doses of raptinal or rotenone and assessed for LDH release at 24h post-treatment.
Figure S2

A

<table>
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<tr>
<th></th>
<th>Raptinal</th>
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<td>0 20 10</td>
</tr>
<tr>
<td>GSDME KO</td>
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B

GSDME WT | GSDME KO

Raptinal (5μM)

C

PI uptake (% Maximal)

D

Rotenone

Plotted % uptake (% Maximal)

E

6-OHDA

Plotted % uptake (% Maximal)

F

Antimycin-A

DMSO

G

3-NP

DMSO

H

LDH Release

I

GSDME WT | GSDME KO

DMSO

J

Thapsigargin

DMSO

K

Tunicamycin

Plotted % PI uptake (maximal)

Plotted % PI uptake (maximal)
Figure S2: GSDME is activated by mitochondrial and ER toxins and mediates cell death in human SH-SY5Y cells (related to Figure 2).

(A) Immunoblots of WT and GSDME KO SH-SY5Y treated with DMSO, raptinal, rotenone or 6-OHDA. Cells treated with raptinal were harvested for lysates at 2h post-treatment, while cells treated with rotenone or 6-OHDA were collected at 12h. All concentrations are in micromolar units.

(B) Representative 20X Images of WT (left) or GSDME KO (right) SH-SY5Y cells at 2h following treatment with 5uM raptinal. Cells were incubated in Sytox Green-containing media, and green cells indicate dye uptake. Scale bar: 200 um

(C-G) WT SH-SY5Y and GSDME KO cells treated with (C) 5uM raptinal (D) 20uM rotenone (E) 30uM 6-OHDA (F) 1uM antimycin or (G) 1mM 3-nitropropionic acid were co-incubated with propidium iodide, imaged every 3h and quantified for PI uptake.

(H) WT or GSDME KO SH-SY5Y cells were incubated in media containing 5uM Raptinal, 20uM rotenone, or 30uM 6-OHDA and assessed for LDH release at 24h post-treatment.

(I) Representative 20X images of wild-type or GSDME knockout SH-SY5Y cells stained with propidium iodide (PI) and treated with DMSO or 0.4uM thapsiagargin (Tg) for 24h.

(J-K) SH-SY5Y were incubated in propidium iodide containing media and treated with (J) 0.4uM thapsiagargin (Tg) or (K) 4uM tunicamycin. Images were taken every 4h in an IncuCyte ZOOM machine and propidium iodide uptake was quantified.
**Figure S3: Caspase-3 but not RIPK1 inhibition protects neurons from mitochondrial toxins (related to Figure 2)**

(A-B) Immunoblots of (A) primary mouse neurons or (B) SH-SY5Y treated with DMSO or raptinal (5-10uM) in the presence or absence of 20uM zVAD-FMK.

(C-D) Representative 20X images of primary neurons stained with PI and treated with either 5uM raptinal or 5uM raptinal + 20uM zVAD-FMK (C). Images were taken every 3h by an IncuCyte ZOOM and PI uptake was quantified (D). Scale bar: 200um

(E-F) Representative 20X images of SH-SY5Y stained with PI and treated with either 5uM raptinal or 5uM raptinal + 20uM zVAD-FMK (E). Images were taken every 3h in an IncuCyte ZOOM and PI uptake was quantified (F). Scale bar: 200um

(G-H) Primary mouse neurons treated with (G) 5uM raptinal or (H) 30uM rotenone were co-incubated in media containing PI and either DMSO, nec1 or GSK ‘872 (RIPK1 inhibitors). Images were taken every 3h and quantified for PI uptake.
Figure S4
Figure S4: GFP-GSDME cleavage by mitochondrial toxins and downstream caspase-3 causes enhanced cell death and puncta formation (related to Figures 2 & 3)

(A) Diagram showing the full-length human GSDME (yellow), and caspase-3 cleavage site (dotted line). An eGFP (green) sequence was placed on the N-terminal side of hGSDME.

(B-C) HEK 293T cells were transfected with GFP (control) or GFP-fl-GSDME and treated with either DMSO or raptinal (5uM). Immunoblots for (B) GSDME, (C) GFP or CASP3 show cleavage and activation of CASP3 and the GFP-GSDME construct.

(D-F) Cells were transfected with GFP-GSDME (top panels) or control vector (bottom panels) and co-incubated with PI and either raptinal or rotenone. Cells were imaged every 1h and quantified for PI uptake (E-F).

(G) Primary WT neurons transduced with GFP-GSDME were incubated with 5uM raptinal and immunoblots were performed with cells treated for different times to assess the temporal dynamics of caspase-3 and GSDME activation.
Figure S5: Mitochondrial toxins induce GFP-tagged GSDME to form puncta in a caspase-dependent manner (related to Figures 3).

(A-B) SH-SY5Y cells treated with raptinal + DMSO, or raptinal + 20uM zVAD, and imaged over 90 minutes. White arrows represent intracellular GFP-GSDME puncta, while arrowheads denote plasma membrane enrichment (A). Images were captured every 10 min and the frequency of cells showing intracellular puncta, as well as positive PI staining were quantified (B). Scale bar: 10um

(C) Primary mouse neurons were co-transfected with mTagRFP-Membrane-1 (neuromodulin: aa 1-20) and GFP-GSDME and treated with 5uM raptinal. Following toxin treatment, images were taken every 30 min (for 10h total) to study the colocalization of these two markers. Scale bar: 20um

(D) Primary neurons transfected with GFP-GSDME were treated with 5uM raptinal or 5uM raptinal + 20uM zVAD-FMK and imaged 45 minutes after drug treatment. Top (large) panels show representative single neurons prior to any drug treatment. Scale bars: 50 um. The bottom panels show magnified axonal segments (white boxes) before and after 45 min of drug treatment. Scale bars: 20 um

(E) Quantification of GFP-GSDME puncta treated with either 5uM raptinal or 5uM raptinal + 20uM zVAD-FMK. Each dot represents a single neuron, and 8-10 neurons from three independent experiments were used for quantification. Data was analyzed using paired Student’s t-tests comparing before and after drug treatment. P-values were adjusted for multiple comparisons using the Tukey method.
Figure S6
Figure S6: Toxin treatment causes GSDME to colocalize with mitochondria (related to Figure 3)

(A-B) Representative cell bodies from (A) mouse neurons transfected with mitochondrial marker mKate-OMP25 (red) and GFP-GSDME (green) before and after 3h treatment with 5uM raptinal are shown. (B) The enrichment of the green signal on mitochondria over cytosol was quantified and compared before and after raptinal treatment (N=12 cell bodies representing neurons from 3 replicate experiments).

(C-E) Mouse neurons transfected with mitochondrial marker mKate-OMP25 or GFP-GSDME were imaged before and after treatment with 20uM rotenone. (C) Representative images of neurons at baseline and 4h post-rotenone treatment. (D) The location of mitochondria (red) and GFP-GSDME (green) peaks were identified along the neurites. (E) The position of the red signal overlapping with green GSDME fluorescence was quantified and compared before and after raptinal treatment.

(F) SH-SY5Y cells transfected with mitochondrial marker mKate-OMP25 or GFP-GSDME were imaged before and after treatment with 5uM raptinal. Images were captured 2.5h post-raptinal treatment.

(G-H) Immunoblot of enriched cytosolic and mitochondrial (ATP5F1 positive) fractions from SH-SY5Y treated with either DMSO or 10uM raptinal for 1h. (G) The top two panels represent full length and cleaved GSDME fragments. (H) Quantification of the intensity of cleaved over full length GSDME bands in immunoblots from cytosolic or mitochondrial SH-SY5Y fractions (n=3).

(I-J) Immunoblot of enriched cytosolic and mitochondrial (ATP5F1 positive) fractions from primary neurons treated with either DMSO or 10uM raptinal for 1h. The top two panels (I) represent full length and cleaved GSDME fragments. (J) Quantification of the intensity of cleaved over full length GSDME bands in immunoblots from cytosolic or mitochondrial primary neuron fractions (n=3).

(K-L) Immunoblots of WT or GSDME KO SH-SY5Y treated with DMSO or 5uM raptinal for 1h. (K) Mitochondrial (ATP5F1) and cytosolic fractions were blotted for GSDME, Cl-CASP3 and cytochrome-c (Cyt-c). The ratio of mitochondrial to cytosolic cytochrome-c signal by immunoblot was (L) quantified for WT and KO cells treated with 5uM raptinal (n=4).
Figure S7: GSDME recruitment to mitochondria is cardiolipin dependent (related to Figure 3)

(A) Primary mouse cortical neurons were transduced with lentiviruses encoding 3 different shRNAs (#1-3) targeting mouse cardiolipin synthase 1 (CLS1) and scrambled control. Immunoblotting against CLS1 revealed efficient KD with shRNA#2.

(B-C) Representative images of GSDME KO mouse neurons transfected with plasmids encoding CLS1-shRNA#2 or scrambled control and co-transfected with both GFP-GSDME and mKate-OMP25. 3d post-transfection, cells were treated with 5uM of raptinal and imaged at 0, 3h and 4h post treatment to quantify GSDME puncta co-localized with mitochondria (C).
Figure S8: Toxins cause GSDME activation and mitochondrial colocalization in human iNeurons (related to Figure 3).

(A-B) Immunoblots of human iPSC-derived cortical neuron (iNeuron) cultures. (A) Immunoblot of human iNeurons treated with 5uM or 10uM raptinal for 1h. (B) Immunoblot of human iNeurons treated with DMSO, 20uM zVAD, 5uM raptinal or a combination of raptinal and zVAD for 1h.

(C-E) Human iPSC-derived cortical neurons transfected with mitochondrial marker mKate-OMP25 and GFP-GSDME were imaged before and after a 2.5h treatment with 5uM raptinal. A representative neuron (C, top panel), with magnified images of a neurite (indicated by dotted white box) before and after raptinal treatment are shown (C, bottom panel). (D) Locations of mitochondria and intensity of GFP-GSDME were measured as line scans along neurites. Line scans (as shown in panel D) were analyzed to calculate (E) GFP-GSDME enrichment on mitochondria before and after raptinal treatment. N >100 axon segments representing iNeurons originating from 3 independent differentiations.
Figure S9: GSDME deficiency protects from TMRM loss in primary neurons and SH-SY5Y (related to Figure 4)

(A-D) Wild-type and Gsdme KO primary cortical neurons were stained with TMRM and incubated with (A-B) 0.5μM and 1μM raptinal or (C-D) 10μM and 20μM antimycin-A. Representative images of TMRM uptake are shown (A and C). TMRM intensity was quantified at 30 min and 1h post-toxin treatment, respectively (B and D).

(E-G) Wild-type and GSDME KO SH-SY5Y were stained with TMRM and incubated with (E-F) 0.5μM or 3μM raptinal or (G) 10μM rotenone. Images captured at 4 and 10h post-treatment were quantified for TMRM intensity (F-G).

All TMRM intensities are represented as intensities to respective DMSO controls.
Figure S10: GSDME deficiency protects from caspase-3 activation and neurite loss (related to Figures 4 & 5).

(A) Representative images at several timepoints of wild-type and GSDME KO SH-SY5Y cells treated with 2uM raptinal and incubated in media containing Incucyte Caspase-3/7 dye.

(B-C) Wild-type and GSDME KO SH-SY5Y incubated in Caspase-3/7 dye were treated with (B) 5uM raptinal and imaged every 3h post-treatment. (C) Area under the curve (AUC) measurements from 5uM raptinal (AUC_{6h}), 20uM rotenone (AUC_{24h}) and 30uM 6-OHDA (AUC_{12h}) treatment were generated and compared between WT and KO cells.

(D-E) Wild-type and Gsdme KO primary neurons incubated in Caspase-3/7 dye were treated with (D) 5uM raptinal and imaged every 2-3h post-treatment. (E) Area under the curve (AUC_{24h}) measurements from 5uM raptinal, 20uM rotenone and 20uM antimycin-A treatment were generated and compared between wild-type and Gsdme KO cells.

(F) Representative images of wild-type neurons treated with DMSO, 3uM raptinal and 20uM antimycin-A and stained for Tuj1 at 8h post-toxin treatment.

(G) Microtubule depolymerization index was calculated for WT neurons treated with several doses of raptinal and antimycin-A. Violin plots display the combined median and interquartile ranges for depolymerization index.

(H) Wild-type and Gsdme KO mouse cortical neurons were treated with 4uM raptinal, 20uM rotenone or 20uM antimycin-A and were assessed for LDH release at 8h post-treatment.

(I) Microtubule depolymerization index was calculated for WT neurons co-incubated with either DMSO or 20uM of zVAD-FMK and were then treated with several doses of raptinal, antimycin-A or rotenone (all concentrations are mentioned in micromolar units). Violin plots display the combined median and interquartile ranges for depolymerization indices taken from two independent experiments.

(J) Representative image of a microfluidic chamber plated with wild-type mouse cortical neurons stained with TMRM. The cell body, microgrooves and axonal compartments are labeled. The top panels represent the chamber prior to addition of 5uM raptinal, while the bottom panels show 1h post-toxin treatment.

(K) Quantification of TMRM intensity relative to baseline (time = 0) from the cell body and axonal chambers of plated WT neurons treated with 5 or 10uM raptinal.

For all datasets two-way ANOVA (row factor = toxin , column factor = genotype) was performed, followed by multiple comparisons for each group (p-values adjusted by the Tukey method are mentioned over respective comparisons). Data represents an average of at least 2 independent experiments.
Figure S11: Expression of N-GSDME in neurons is sufficient to drive mitochondrial localization, mitochondrial damage and neurite loss (related to Figure 5)

(A) Representative widefield images of mouse neurons transfected with mKate2-OMP25 and either full-length GFP-GSDME (top row), N-terminal GSDME (middle row) or empty vector control (bottom row). Cells were imaged 16h post-transfection.

(B) Structured illumination microscopy (SIM) images of mitochondria (OMP25-mKate) in axons of mouse neurons transfected with GFP-N-GSDME to assess colocalization.

(C) Representative widefield images of SH-SY5Y transfected with mKate2-OMP25 and either full-length GFP-GFP-N-GSDME (top image) or GSDME (bottom image).

(D) Structured illumination microscopy (SIM) images of SH-SY5Y mitochondria (mKate-OMP25) transfected with GFP-N-GSDME to assess colocalization.

(E-F) Primary mouse neurons (DIV3) were transduced with lentivirus encoding either N-GSDME or GFP-control. (E) 4d post-transduction (DIV7) neurons were stained with TMRM. (F) Images from 6 wells per condition across 2 independent experiments were quantified by measuring TMRM intensity along the length of neurites.

(G-H) Primary mouse neurons (DIV3) were transduced with lentivirus encoding either N-GSDME or GFP-control. (G) 4d post-transduction, neurons were fixed and stained for Tuj1+ signal. (H) Images from 6 wells per condition across 2 independent experiments were used to calculate microtubule depolymerization index for each condition.

For datasets F, H one-way ANOVA was performed, followed by multiple comparisons for each group (adj p-values, Tukey method). For F,H data represents an average of 2 independent experiments (3 technical replicates/experiment) ± SEM.
Figure S12: GSDME is activated in iNeurons and mediates neurite loss caused by FTD/ALS proteins (related to Figure 6)

(A) GSDME immunoblot quantification (n=5-6) of primary cortical neurons transduced with lentivirus encoding GFP, PR-50, TD-43 or un-transduced control. Four days following transduction with lentiviruses, samples were lysed and probed with anti-GSDME and GAPDH (loading control). The ratio of N-terminal GSDME to full-length was calculated and plotted relative to untreated control.

(B) Representative images of WT and Gsdme KO mouse cortical neurons stained with TMRM (red) and transduced with lentiviruses encoding PR-50 or TDP-43 (4d post-transduction).

(C-D) Human iNeurons were transfected with mKate-OMP-25, GFP-GSDME and TDP-43 or iRFP (control). (C) Cells were imaged 72h post-transfection (D) Quantification of the number of GFP-GSDME puncta per 100um in neurites.

(E) Human iNeurons were transduced with control virus (GFP) or lentivirus encoding FLAG-TDP43 at multiplicity of infection (MOI) of 4 or 8. 5d post-transduction, cells were lysed for immunoblot analysis.

(F-I) Primary wild-type and Gsdme KO mouse neurons were co-transfected with RFP (shown in black) and either GFP, (F-G) TDP-43 or (H-I) PR-50 and imaged for neurite (RFP signal) area. Representative images of WT and KO neurons transfected with GFP and either TDP-43 (F) or PR-50 (H) are shown along with the quantifications of neurite area (G and I). Each dot represents an average of 10-12 transfected neurons imaged in an independent well. Every condition was imaged across two independent experiments.

For datasets A, G, I two-way ANOVA (row factor = lentivirus treatment, column factor = genotype) was performed, followed by multiple comparisons for each group (adj p-values, Tukey method). For F-I, data represents an average of 2 independent experiments ± SEM.
Figure S13: Transcriptomic analysis of human spinal cord, and validation of iPSC-derived motor neurons (related to Figure 7)

(A) RNA-seq transcriptomic data from laser capture micro-dissected motor neurons from patients with sporadic ALS and age-matched controls.

(B) Sanger sequencing chromatograms of 1016A (WT) and TDP43^{G298S} iPSC-derived motor neurons at the TDP-43 locus.

(C) G-banded karyotypes of 1016A (WT) and TDP43^{G298S} iPSC-derived motor neurons.

(D) Phase contrast imaging of iPSC morphology from 1016A (WT) and TDP43^{G298S} cell lines.

(E) Phase contrast imaging of embryoid body morphology from 1016A (WT) and TDP43^{G298S} cell lines.

(F) Immunostaining of 1016A (WT) and TDP43^{G298S} iPSC-derived motor neurons positive for Islet 1/2 (motor neuron marker), co-stained with Tuj1, and then treated with MG-132, thapsigargin and tunicamycin for 48h.
Figure S14

A. Vehicle, MG132 (1uM), Thapsigargin (0.5uM), Tunicamycin (5uM) on WT and TDP43

B. MG132

C. Thapsigargin

D. Tunicamycin

E. GSDME

F. GSDME levels (Fold Change)
Figure S14: Endoplasmic reticulum stressors and proteasome inhibition cause neurite loss in susceptible TDP43 G298S iPSC-derived motor neurons (related to Figure 7)

(A) Representative images of control 1016A (WT) or TDP43<sup>G298S</sup> iPSC-derived motor neurons treated with DMSO, MG132, tunicamycin or thapsigargin, and stained for Tuj1 at 48h post-toxin treatment.

(B-D) Microtubule depolymerization index was calculated for 1016A (WT) and TDP43<sup>G298S</sup> motor neurons treated with DMSO (vehicle) or several doses (C) MG132 (D) thapsigargin or (E) tunicamycin (uM). Violin plots display the combined median and interquartile ranges for depolymerization index.

(E-F) Immunoblots of iPSC-derived cortical neurons 4d post-transduction with scrambled or GSDME-targeting shRNAs (E), which was quantified in (F).
Figure S15: GSDME is activated in the SOD1 G93A mouse model and plays a role in disease progression (related to Figure 8)

(A-B) GSDME and GAPDH immunoblots of spinal cord lysates from (A) pre-symptomatic stage (P82) and end-stage (P160) SOD1<sup>G93A</sup> mice. (B) The activation ratio of GSDME is quantified as the levels of GSDME N-terminal compared to total GSDME (full length + cleaved fractions).

(C) Expression of the SOD1<sup>G93A</sup> transgene, as assessed by RT-qPCR, was quantified for each group and genotype. This was done to confirm that the copies of this ALS-causing transgene were comparable between experimental groups.

(D) Weights of transgenic SOD1<sup>G93A</sup> Gsdme WT or SOD1<sup>G93A</sup> Gsdme KO mice were tracked over time starting at week 7 until week 21.

(E) Grip strength measures of nTg WT and nTg Gsdme KO mice (lacking SOD1<sup>G93A</sup>) were recorded at P60 and P150 and normalized by body weight.

(F) Table of summary values for male and female transgenic mice displaying key measures of survival, disease progression and motor function.
Figure S16: GSDME knockout reduces histological markers of gliosis in the SOD\textsuperscript{1G93A} mouse model of ALS (related to Figure 8)

(A-B) Representative images of lumbar spinal cord sections from SOD\textsuperscript{1G93A} Gsdme WT and SOD\textsuperscript{1G93A} Gsdme KO animals at P150 (A) stained with anti-GFAP to mark astrocytes. (B) The intensity of GFAP in the ventral horn of P150 transgenic mice normalized by the ventral horn area was quantified. Each dot represents the average value of 6-8 stained lumbar spinal cord sections taken from n=3-5 mice. Magnified regions (white box) delineate a ventral horn area of a spinal cord section.

(C-D) Representative images of lumbar spinal cord sections from SOD\textsuperscript{1G93A} Gsdme WT and SOD\textsuperscript{1G93A} Gsdme KO animals at P150, stained with anti-Iba1(C). Magnified regions represent the ventral horns of the spinal cord used for (D) quantification of mean Iba1 counts per um\textsuperscript{2}. Each dot represents the average value of 6-8 stained lumbar spinal cord sections taken from n=3-5 mice.

(E-F) Representative images of lumbar spinal cord sections from SOD\textsuperscript{1G93A} Gsdme WT and SOD\textsuperscript{1G93A} Gsdme KO animals at P150, stained with anti-CD68 (a lysosomal activation marker), to assess disease associated inflammation (E). Magnified regions represent the ventral horns of the spinal cord used for (F) quantification of mean CD68 counts per um\textsuperscript{2}. Each dot represents the average value of 6-8 stained lumbar spinal cord sections taken from n=3-5 mice.
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<th>Clinical Diagnosis</th>
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**Table S1: Patient characteristics of human temporal lobe cortical tissue sections.** NC = normal controls, with patient IDs and represented in images shown in the manuscript (Related to Fig 1).
Table S2: Oligonucleotides and virus details (Related to STAR Methods Key Resources Table)

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Table S3: Donor information for iPSC derived motor neurons (Related to Fig 7).

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<td>Alami et al., 2014</td>
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Supplementary References
