

## Supplementary Information

### Retromer stabilization results in neuroprotection in a model of Amyotrophic Lateral Sclerosis

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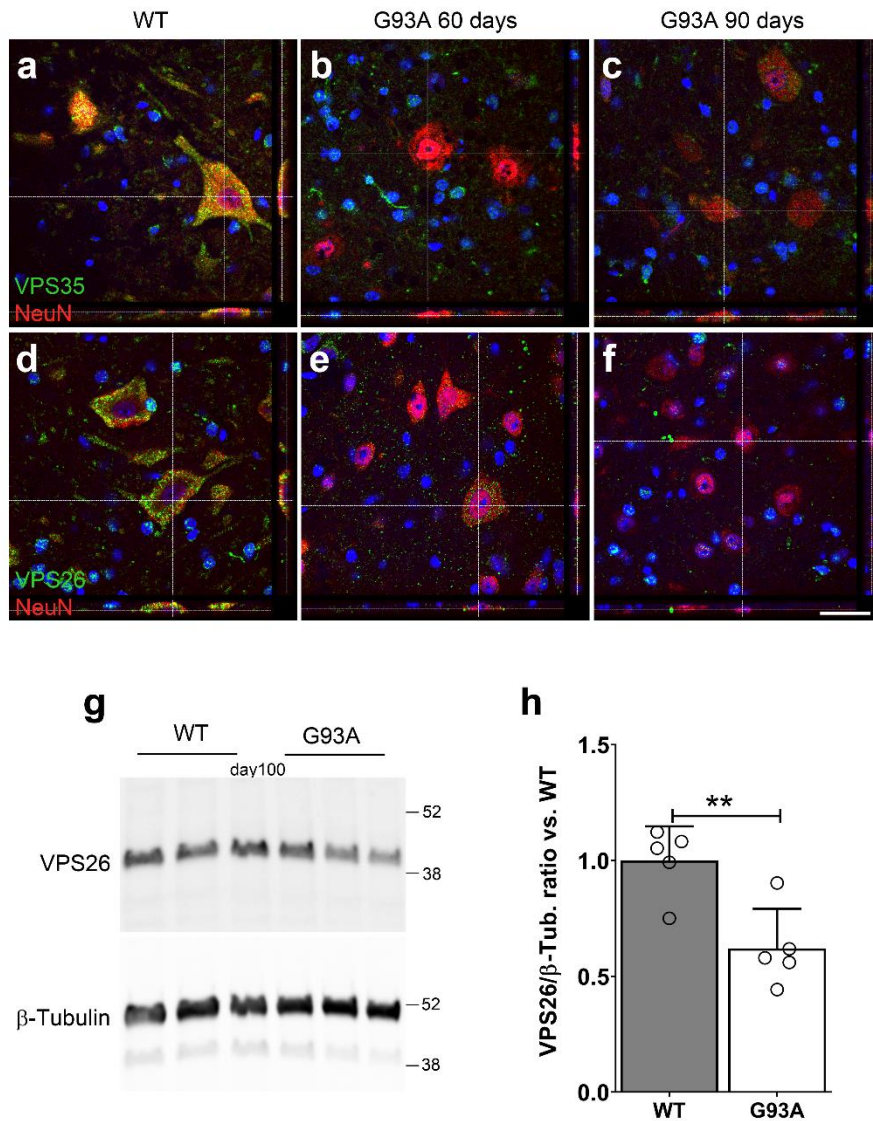
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This PDF file includes:

Supplementary figures 1 to 15

# Supplementary Figure 1

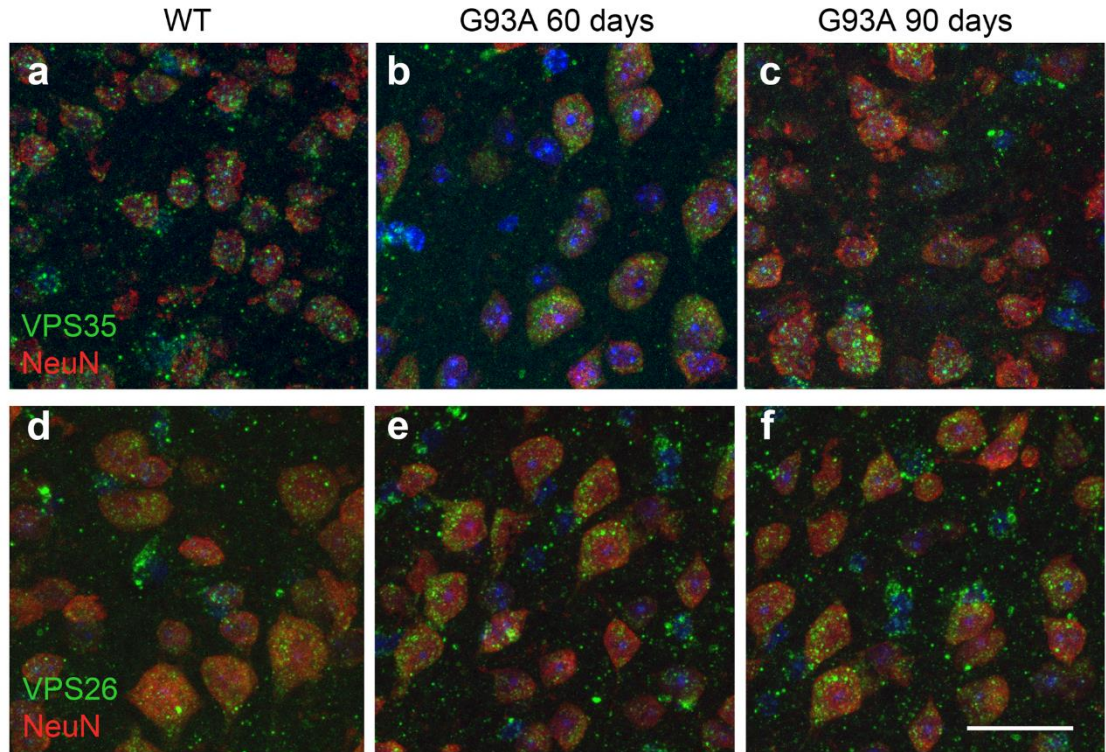
## VPS35/26 distribution in ventral horn MNs of G93A mice



Representative cross sections of confocal stacks acquired with a step of 0.3  $\mu$ m from WT (a, d) and G93A (b, c, e and f) sampled at 60 and 90 days, respectively. a-c show sections labelled for VPS35 and NeuN (n=3 independent mice/group). d-f show sections labelled for VPS26 and NeuN (n=3 independent mice/group). Imaging was done in the ventral horn of the lumbar SC; orthogonal views show the distribution of VPSs<sup>+</sup> in single NeuN<sup>+</sup> MNs (data were examined over 3 independent experiments). Panel g shows a representative WB of lumbar SC protein extracts from WT and G93A mice sampled at day 100 and probed with anti-VPS26 antibody and  $\beta$ -Tubulin; h, quantifications (means  $\pm$  SD) of normalized VPS26 levels (ratio versus WT, n=5 independent mice/group from 2 independent experiments, p=0.0056). Two-tailed Student's test was used to determine the statistical significance of data, \*\* p<0.01. Scale bar 20  $\mu$ m.

## Supplementary Figure 2

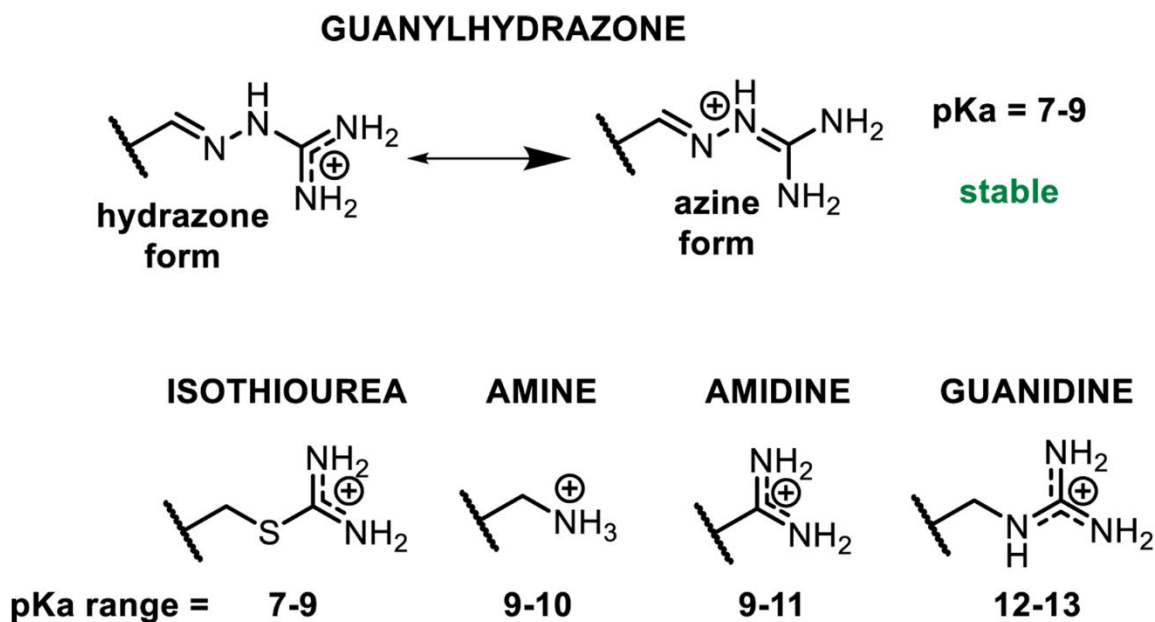
### VPS35/26 in the motor cortex of G93A mice



a-c, representative maximal projections of confocal stacks from WT (a, d) and G93A (b, c, e and f, 60 and 90 days). Imaging was done in the motor cerebral cortex labelled for VPS35 and NeuN, (n=3 independent mice/group, a-c). Adjacent sections (d-f) were labelled for VPS26 and NeuN and images are shown in panels d-f, (n=3 independent mice/group). Data are representative of 2 independent experiments. Scale bar 60  $\mu$ m.

## Supplementary Figure 3

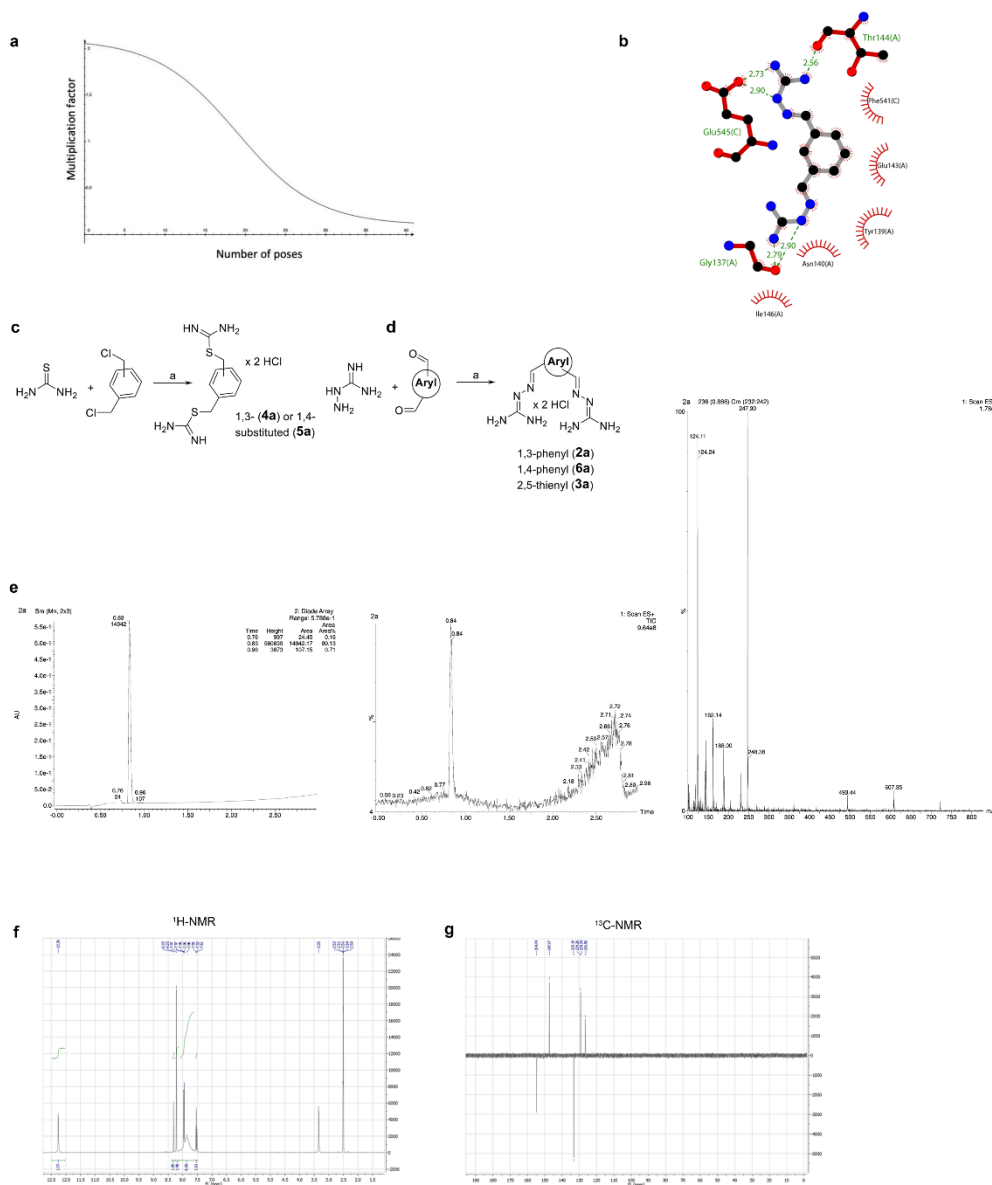
pKa ranges for isothioureas and guanylhydrazones



The figure shows positively charged N-containing groups indicating their pKa range

# Supplementary Figure 4

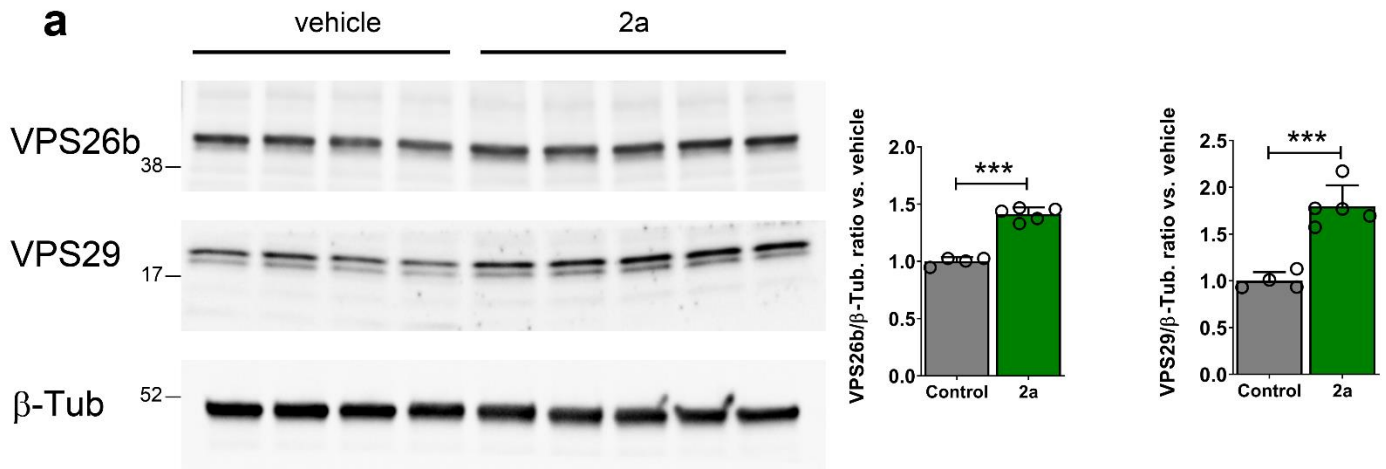
## Synthesis and analytical characterization of compound 2a



a, sigmoidal rectification function based on best pose recurrence in 40 independent runs; b shows the 2D interaction map of 2a. Ligand and protein atoms are represented in ball and stick, evidencing hydrogen bond distances (green lines) and hydrophobic interactions (red semicircles); c Isothioureas 4a and 5a were synthesized as dihydrochloride salts from the corresponding bis-chloromethyl benzenes and thiourea in good yields; d. bis-guanylhya zones 2a, 3a and 6a were synthesized as dihydrochloride salts from the corresponding dialdehydes and aminoguanidine hydrochloride in good yields. e shows the diode Array LC trace (right panel), the TIC trace (middle panel), and an ES scan (left panel, 0.866 peak) for compound 2a; f and g show the  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra of pure compound 2a, respectively.

## Supplementary Figure 5

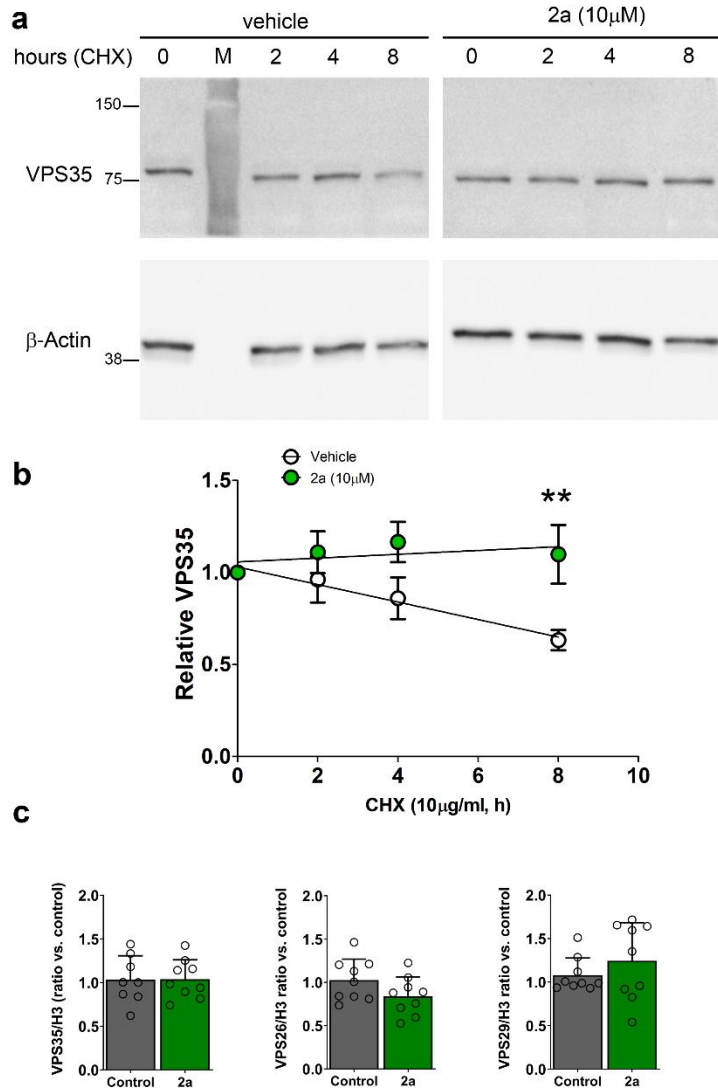
### Compound 2a increases VPS26b and VPS29 in Neuro2a cells



a, WBs for VPS26b, VPS29 and  $\beta$ -Tubulin in vehicle- and compound 2a-treated Neuro2a cells (10  $\mu$ M, 48h,  $3 \times 10^5$  cells/well). Quantitative analyses of blots (means  $\pm$  SD) are shown in histograms (control: n=4 independent wells, 2a: n=5 independent wells examined in one experiment,  $p < 0.0001$  for all the comparisons. Two-tailed Student's test was used to determine the statistical significance of data, \*\*\*  $p < 0.0001$

## Supplementary Figure 6

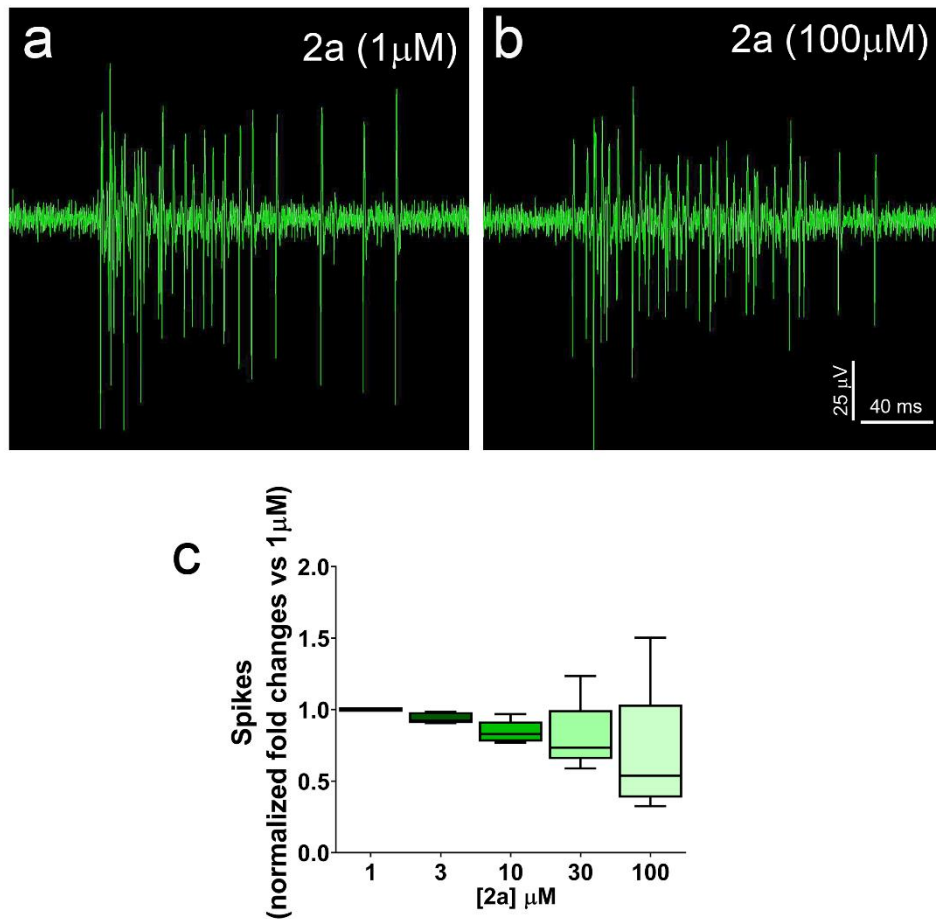
### Compound 2a stabilizes VPS35 levels in Neuro2a cells



A cycloheximide (CHX) chase assay was performed to determine the degradation rate of VPS35 in Neuro2a cells ( $3 \times 10^5$  cells/well). Cells were treated with vehicle or 2a (10  $\mu$ M) for 24 h, and then chased with CHX (10  $\mu$ g/mL) for 2, 4 and 8 h. Panel a shows representative WBs for VPS35 and  $\beta$ -Actin levels. Quantifications of normalized VPS35 levels (means normalized ratios  $\pm$  SEM versus the time 0) are shown in panel b (vehicle:  $n=10$  independent wells, 2a:  $n=8$  independent wells obtained from 3 independent experiments; control vs. 2a at 8 hours:  $p=0.009$ ). c, quantitative real time PCR for *Vps35*, *Vps26* and *Vps29* mRNA (Vps35: controls  $n=8$  independent wells; 2a:  $n=9$  independent wells; Vps26 and Vps29:  $n=9$  independent wells/group); data were collected from 3 independent experiments. Compound-2a was incubated at 10  $\mu$ M for 48h ( $3 \times 10^5$  cells/well); histograms report the ratio versus vehicle-treated cells (means  $\pm$  SD). Two-way ANOVA followed by Bonferroni multiple comparisons test was used to analyze data plotted in panel b. Two-tailed Student's test was used to determine the statistical significance of data plotted in panel c. \*\*  $p<0.01$ .

## Supplementary Figure 7

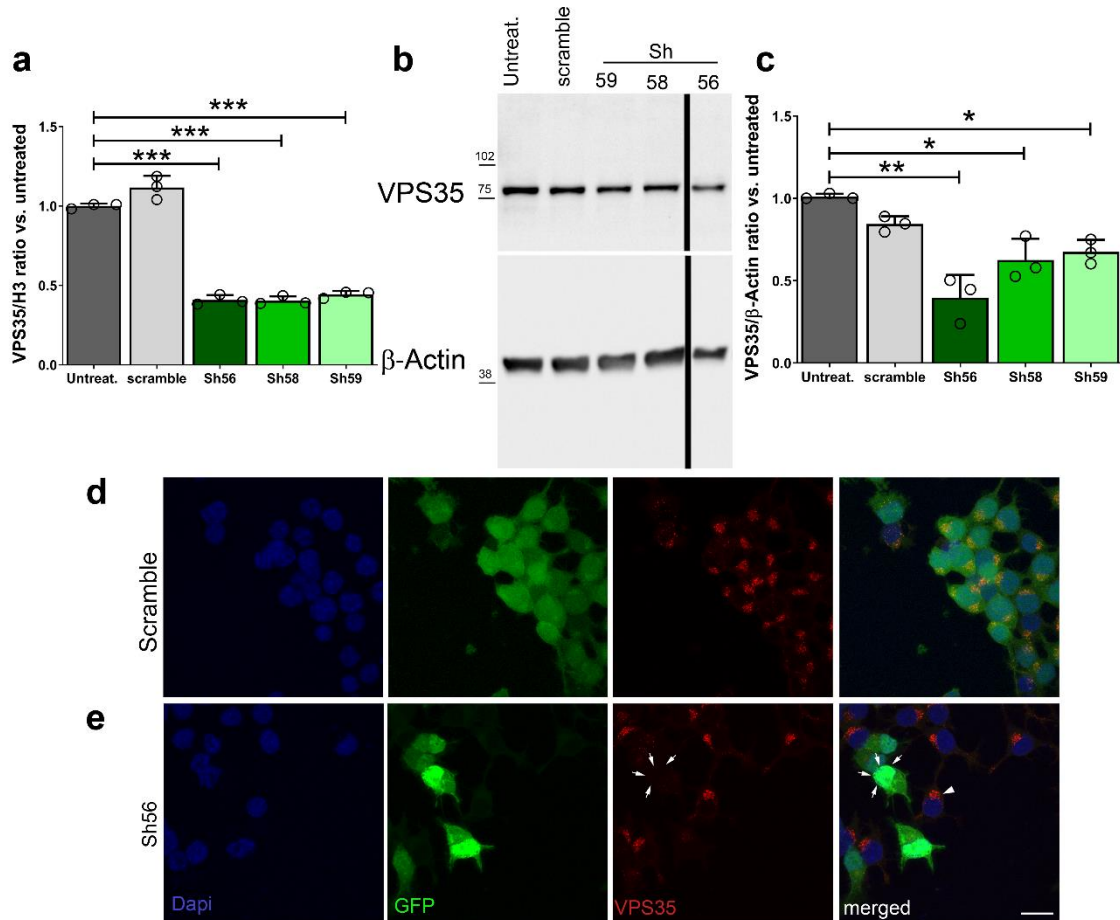
Compound 2a (10 $\mu$ M) does not perturb neuronal firing.



Panels a and b; neuronal activity from the same electrode of a representative MEA biochip ( $3 \times 10^5$  cortical neurons/chip,  $n=5$  independent chips from 2 independent experiments) treated with 2a, at the concentration of 1  $\mu$ M (a) and 100  $\mu$ M (b). Neurons received increasing amounts of lead 2a (1, 3, 10, 30 and 100  $\mu$ M) and were recorded every 5 min; c, quantifications of the firing activity reported as fold changes of spike numbers (box plots show median values; whiskers show 10-90 percentile) measured at the concentration of 1  $\mu$ M. Kruskal-Wallis test followed by Dunn's multiple comparison test was used to analyze the data.

## Supplementary Figure 8

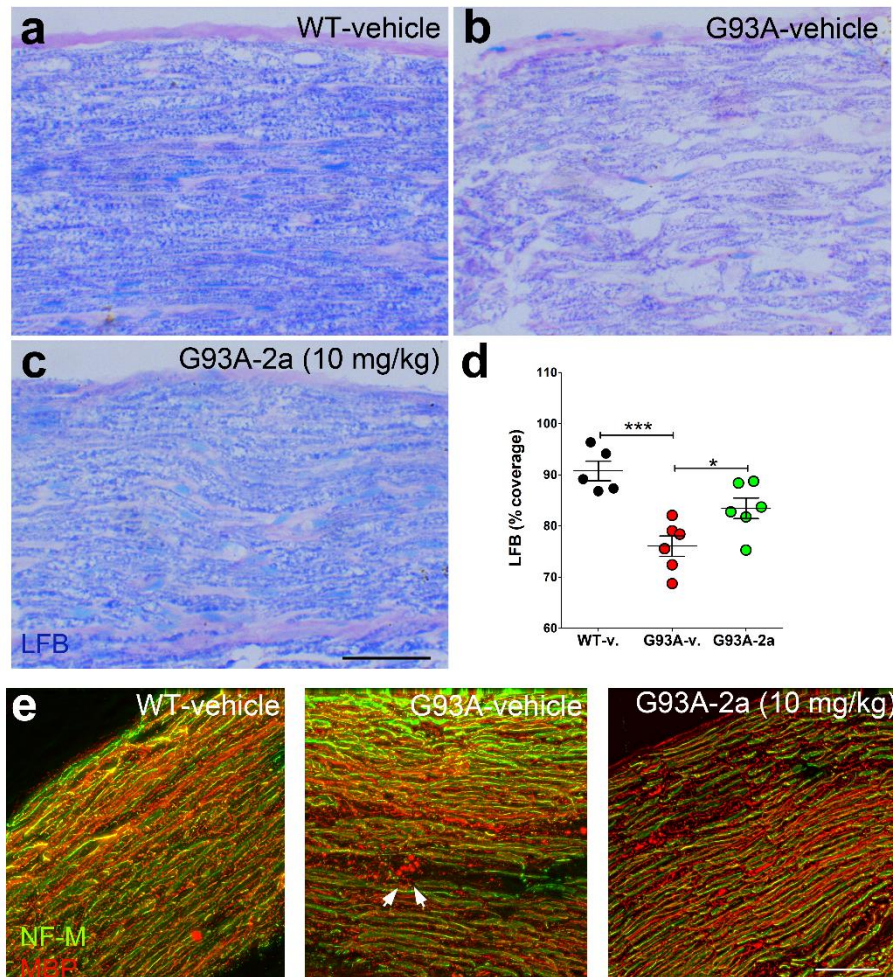
Sh56 short interference sequence efficiently depletes VPS35.



Neuro2a cells ( $3 \times 10^5$  cells/well) were transfected with scramble or with VPS35 RNAi plasmids (Sh56, Sh58 and Sh59,  $2 \mu\text{g}/\text{well}$ ). Knock down was assessed by real time PCR analysis of RNAs sampled 48 h after the transfection. The histogram in panel a shows normalized *Vps35* mRNA levels (mean  $\pm$  SD) reported as ratio versus untreated cells ( $n=3$  independent wells/group from one experiment,  $p<0.0001$  for all comparisons). WBs were carried out to verify VPS35 knockdown. Dividing black lines in panel b indicate cropped lanes from the same filter. Quantifications of normalized VPS35 protein levels (mean  $\pm$  SD) reported as ratio versus untreated cells are shown in panel c ( $n=3$  independent wells/group from 2 independent experiments, untreat. vs. sh56:  $p=0.006$ , untreat. vs. sh58:  $p=0.03$ , untreat. vs. sh59:  $p=0.02$ ). Parallel cultures ( $3 \times 10^5$  cells/well) were transfected with pCAAG-GFP plasmids, along with scramble (d) or with Sh56 (e) plasmids and labelled for GFP and VPS35. Arrows in panel e show VPS35 immunoreactivity in a GFP<sup>+</sup> cells receiving Sh56 plasmids. The arrowhead in panel e indicates a GFP<sup>-</sup> cell from the same field that express VPS35 at normal levels ( $n=3$  wells/group, data derived from 2 independent experiments). One-way ANOVA followed by Tukey's Multiple Comparison test was used to analyze data. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ . Scale bar 10  $\mu\text{m}$ .

# Supplementary Figure 9

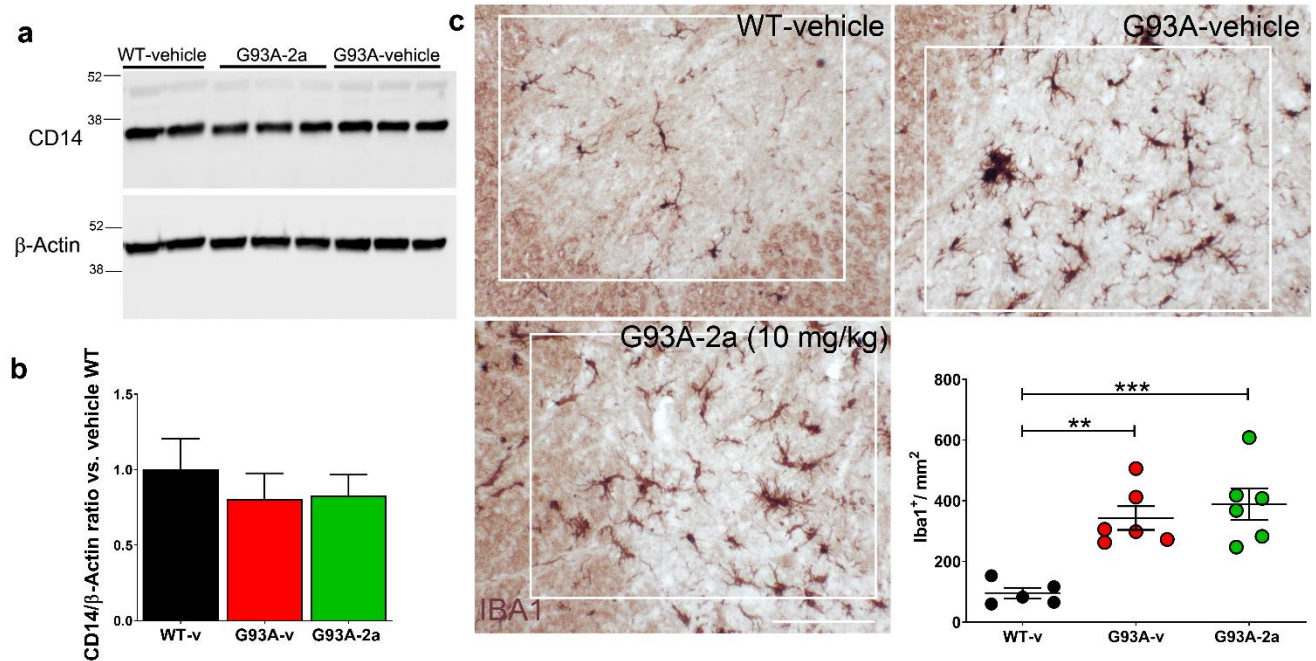
## Compound 2a reduces myelin clearance in G93A mice



Panels a-c, Luxol Fast Blue (LFB) staining on sagittal sections of sciatic nerves from vehicle-treated WT mice (a), vehicle-treated G93A mice, (b) and compound 2a-treated G93A mice (c). Quantifications of (%  $\pm$  SD) LFB covered areas are reported in panel d (WT-v: n=5 independent mice, G93A-v: n=6 independent mice; G93A-2a: n=6 independent mice, data are collected from 2 independent experiments), (WT-v vs G93A-v:  $p < 0.0001$ , G93Av vs. G93A-2a:  $p = 0.016$ ). Parallel sections labelled for NF-M and MBP are shown in panel e (WT-v: n=5 independent mice, G93A-v: n=6 independent mice; G93A-2a: n=6 independent mice, data are collected from 2 independent experiments). Arrows in vehicle treated G93A mice show damaged myelin. One-way ANOVA followed by Tukey's Multiple Comparison test was used to analyze data of panels D. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ . Scale bar 50  $\mu$ m.

## Supplementary Figure 10

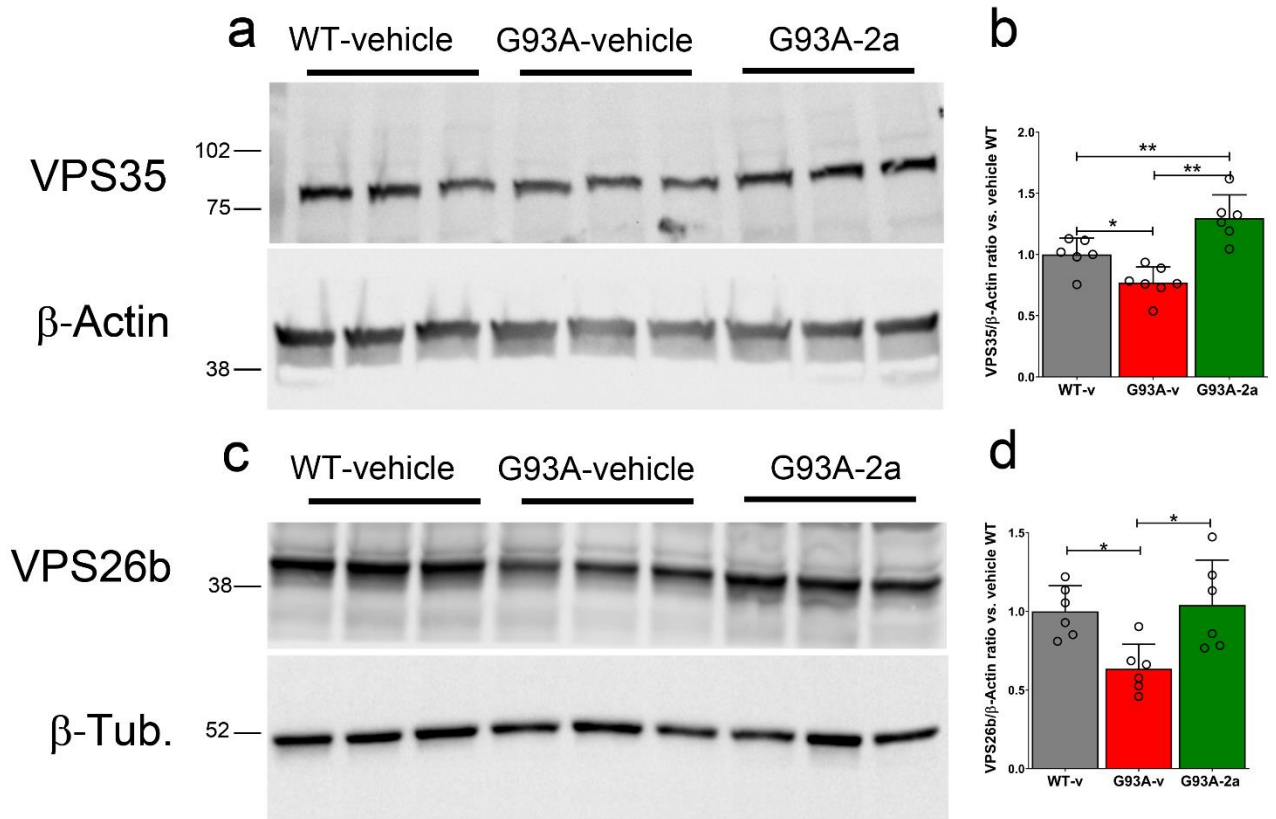
### Compound 2a does not modulate microglia/macrophage in G93A mice



a shows a representative WB for CD14 in lumbar SCs protein extracts from vehicle-treated WT mice, vehicle-treated G93A mice and compound 2a (10 mg/kg)-treated G93A mice (sampled at day 100). Quantifications of normalized CD14 (mean  $\pm$  SD) reported as ratio versus vehicle-treated WT mice are shown in b (n=3 independent mice/group from 2 WB experiments). Panel c shows IHC for Iba1 in vehicle-treated WT mice (n=5 independent mice), vehicle-treated G93A (n=6 independent mice) mice and compound 2a-treated G93A mice (n= 6 independent mice;10 mg/kg, day 100). Cell counts were done in a boxed region (320X260 $\mu$ m) that overlies the ventral horn of the SC (data derived from 2 independent experiments). The histogram in c shows quantifications (lines show means  $\pm$  SEM, WT-v vs G93A-v: p=0.0019, WT-v vs. G93A-2a: p<0.0001). One-way ANOVA followed by Tukey's Multiple Comparison test was used to analyze data plotted in panel b and in panel c. \*\* p<0.01, \*\*\* p<0.001. Scale bar 50 $\mu$ m.

# Supplementary Figure 11

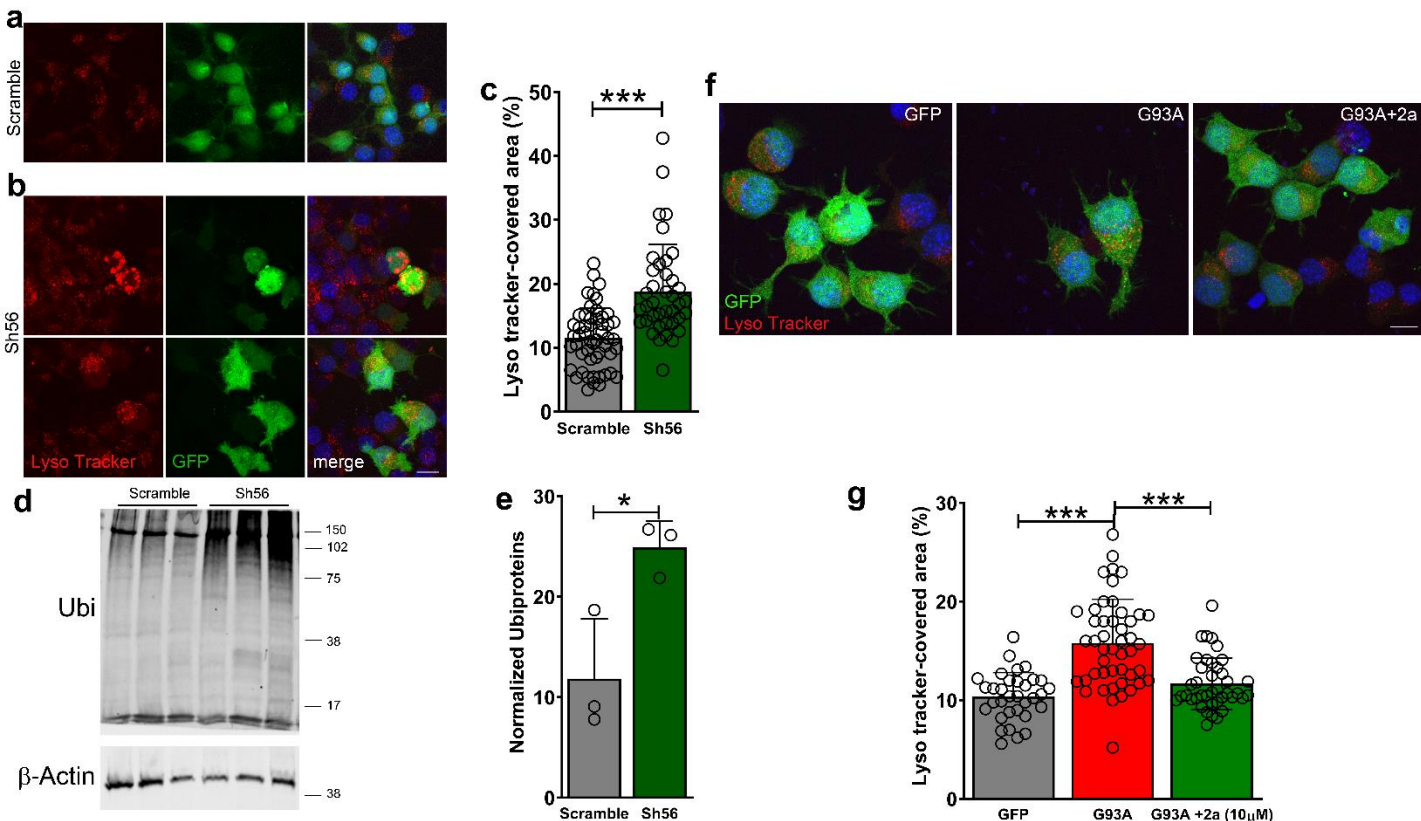
## Compound 2a increases VPS35 levels in G93A mice



Panel a, representative WB for VPS35 and  $\beta$ -Actin in SCs from vehicle-treated WT mice, vehicle-treated G93A mice and compound 2a (10 mg/kg, sampled at day 100)-treated G93A mice (WT-v: n=6 independent mice, G93A-v: n=7 independent mice, G93A-2a: n=6 independent mice). Quantifications of normalized VPS35 levels reported as ratio versus vehicle-treated WT (mean  $\pm$  SD) are shown in panel b (data are collected from 2 independent experiments; WT-v vs. G93A-v: p=0.015, G93A-v vs. G93A-2a: p=0.002, WT-v vs. G93A-2a: p=0.0038). Panel c, representative WB for VPS26b and  $\beta$ -Actin in SC lysates obtained from vehicle-treated WT mice, vehicle-treated G93A mice and lead 2a (10 mg/kg, sampled at day 100)-treated G93A mice (n=6 independent mice/group). Quantifications of normalized VPS26b levels reported as ratio versus vehicle-treated WT (mean  $\pm$  SD) are shown in panel d (data are derived from 2 independent experiments, WT-v vs. G93A-v: p=0.03, G93A-v vs. G93A-2a: p=0.02). One-way ANOVA followed by Tukey's Multiple Comparison test was used to analyze data plotted in b and d. \* p<0.05, \*\* p<0.01.

# Supplementary Figure 12

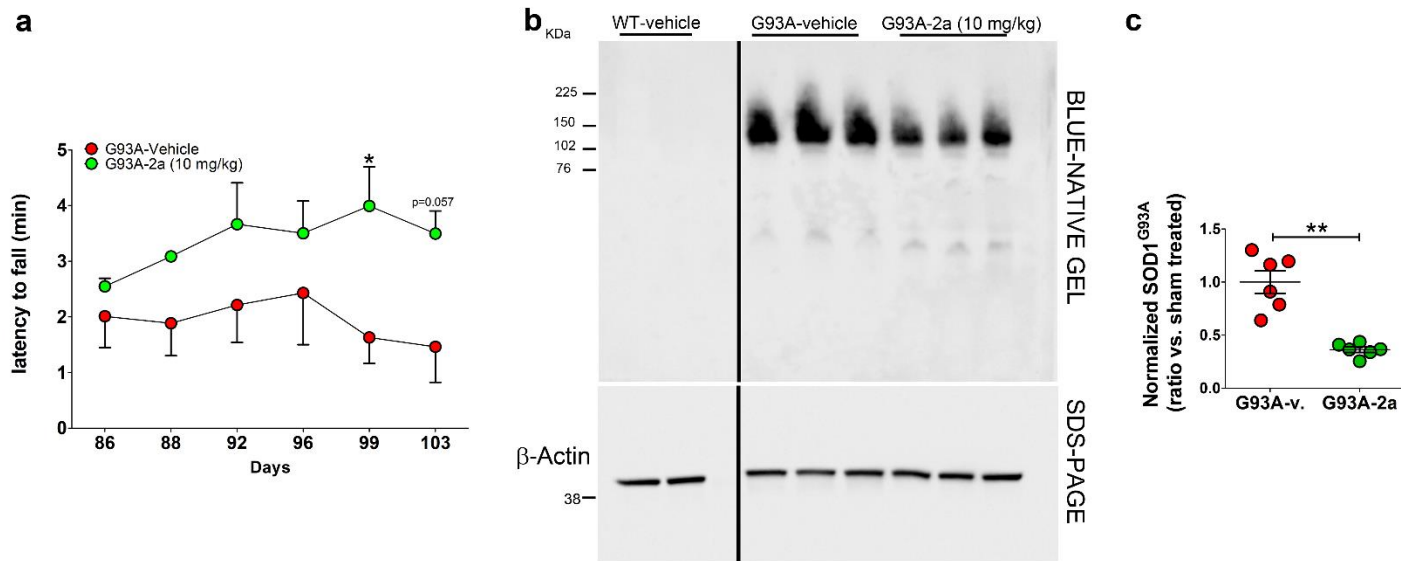
## VPS35 knockdown impairs lysosomal stability



Panels a and b, Neuro2a cells ( $4 \times 10^4$  cells/well) transfected with the scramble plasmid (0.5  $\mu$ g/well, a) or with the VPS35 RNAi plasmids (Sh56, 0.5  $\mu$ g/well, b) along with plasmid encoding the GFP (0.1  $\mu$ g/well) and labelled with Lyso-tracker (75 nM). Quantifications of Lyso Tracker-covered areas ( $\% \pm$  SD) in individual GFP<sup>+</sup> cells were done on merged confocal images and data are shown in c (scramble: n=52 cells, Sh56: n=39 cells, data are collected from 3 independent experiments,  $p < 0.0001$ ). Protein lysates from Neuro2a cells ( $3 \times 10^5$  cells/well) receiving scramble or Sh56 plasmids (2  $\mu$ g/well for 48h) were labelled for Ubiquitin and  $\beta$ -Actin (d). The collective density of each Ubiquitinated-proteins lane was expressed relative to  $\beta$ -Actin levels and mean values  $\pm$  SD are plotted in the histogram of panel e (n=3 independent well/group data are collected from 1 experiment,  $p = 0.025$ ). Neuro2a cells ( $4 \times 10^4$  cells/well) were transfected with plasmid encoding the GFP (0.6  $\mu$ g/well) or with the GFP (0.1  $\mu$ g/well) and pcDNA3.1(+) SOD1<sup>G93A</sup> (0.5  $\mu$ g/well)  $\pm$  2a (10  $\mu$ M) or vehicle. and 24 hours later they were starved and kept in FBS-free-medium  $\pm$  2a for additional 24 h. Cells were incubated with Lyso-tracker (75 nM). Panel f shows a representative merged confocal images of cells labelled for the GFP and the Lyso-tracker. Quantifications of Lyso-tracker-covered areas ( $\% \pm$  SD) in each group are shown in panel g (GFP: n=33 cells, G93A: n=47 cells, G93A+2a: n=40 cells, data are collected from 3 independent experiments,  $p < 0.0001$  for all comparisons). Two-tailed Student's test was used to determine the statistical significance in panels c and e. One-way ANOVA followed by Tukey's Multiple Comparison test was used to analyze data plotted on panel g. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ . Scale bar 10  $\mu$ m.

# Supplementary Figure 13

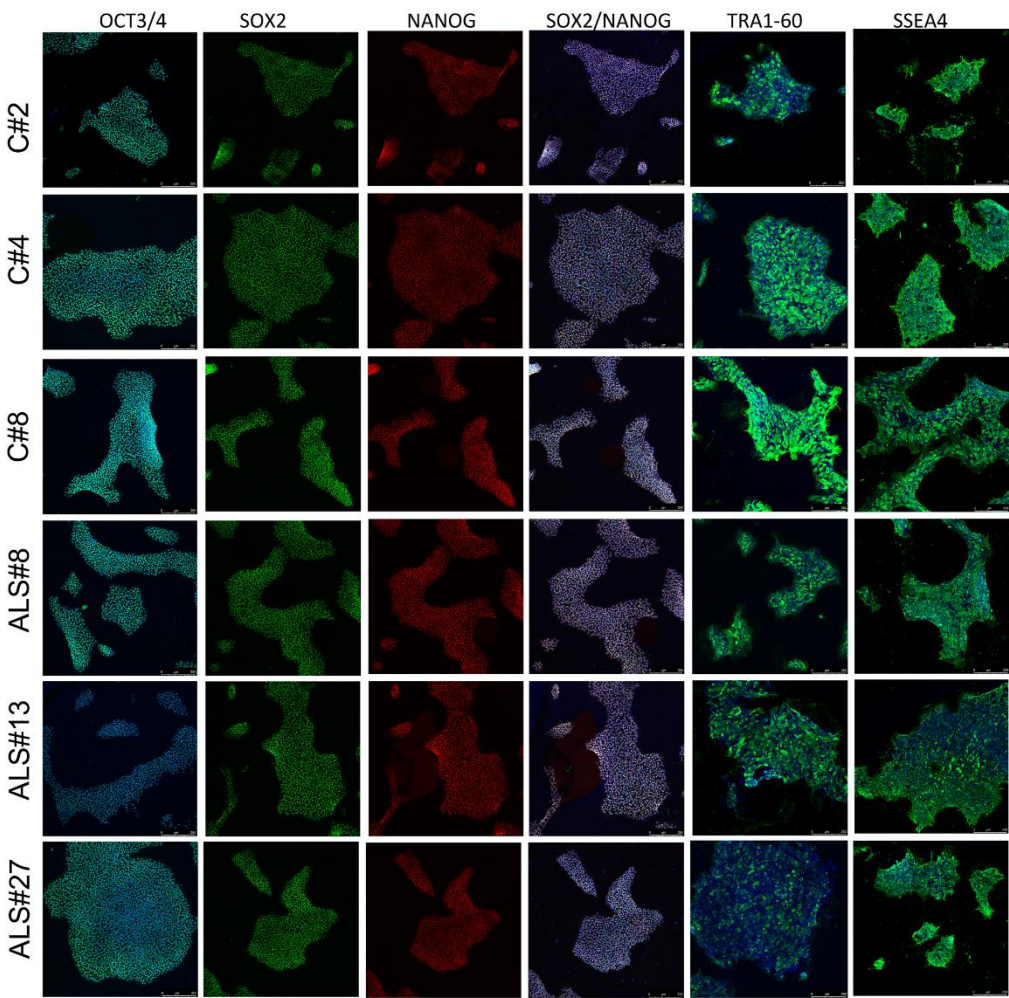
## Compound 2a attenuates SOD1 aggregates in G93A mice



Panel a shows the latency to fall in vehicle- and compound 2a-treated G93A mice (10 mg/kg, n=4 independent mice/group, data are collected from 1 experiment). Data are reported as mean  $\pm$  SD. A representative immuno-blot of a blue-native polyacrylamide gel loaded with lumbar SC extracts from vehicle- and 2a-treated mice is shown in b. Membranes were incubated in acetic acid to fix the proteins and probed with an anti-huSOD1 antibody. Equal amounts of protein extracts were loaded on parallel SDS-PAGE, blotted on nitrocellulose filters and probed with an anti  $\beta$ -Actin antibody. Dividing black lines mark lanes cropped from the same filter; c, quantifications of the mutant forms of the SOD1 from mice treated with sham or with compound 2a (G93A-v: n=6 independent mice, G93A-2a: n=6 independent mice, data are derived from 2 independent experiments). Two-tailed Student's test was used to determine the statistical significance in a ( $p=0.011$ ); the Mann Whitney test was used to determine statistical significance in c ( $p=0.0022$ ). \*  $p<0.05$ , \*\*  $p<0.01$

Supplementary Figure 14

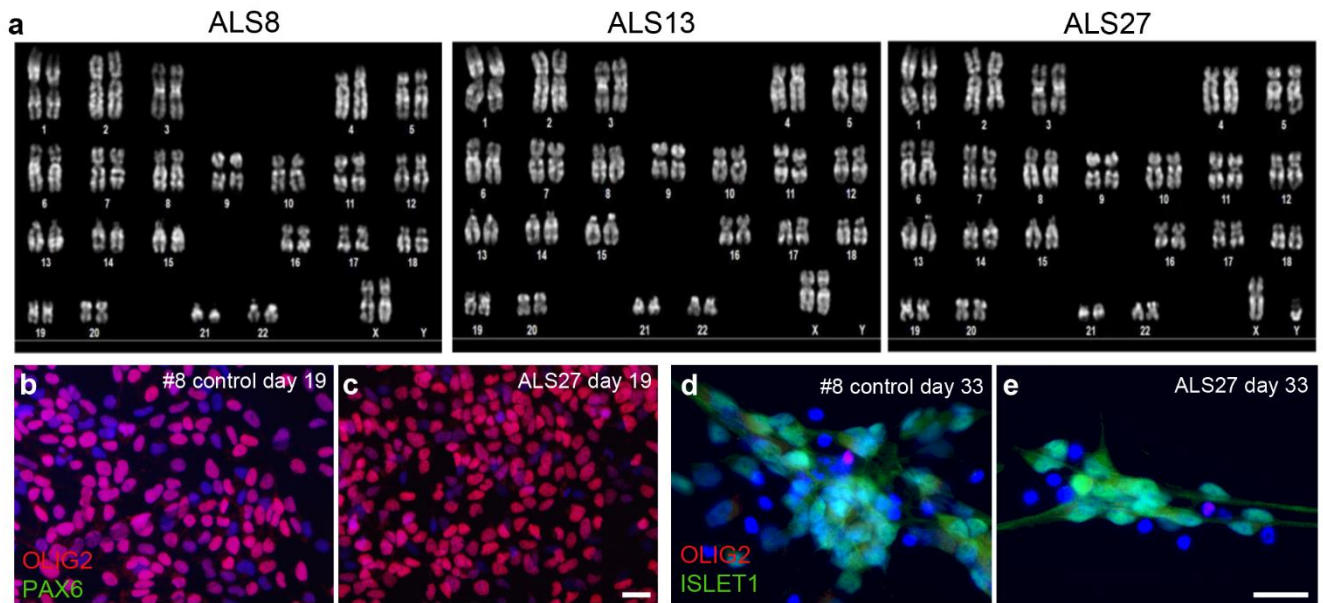
Characterization of iPSC lines



IF analysis of iPSC cell lines for the following pluripotency markers: OCT4, SOX2, NANOG, TRA1-60, SSEA4. Data are derived from 1 experiment. Scale bar 250  $\mu$ m.

# Supplementary Figure 15

## Karyotype and MN differentiation of iPSC lines



Panel a, high –resolution analysis of karyotypes of ALS iPSC lines indicating a normal chromosomal content (data are derived from one experiment). Panel b and c, representative IF for OLIG2 and PAX6 in control (b) and ALS (c) cultures collected 19 days after the induction of the MN differentiation protocol (data are representative of 3 independent experiments). Panel d and e, representative IF for OLIG2 and ISLET1 in control (d) and ALS (e) cultures at the end of the MN differentiation protocol (data are representative of 3 independent experiments). Scale bar 20 μm.