Hyperactive somatostatin interneurons contribute to excitotoxicity in neurodegenerative disorders

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ALS is a fatal neurodegenerative disorder that is characterized by progressive loss of motor neurons in both the motor cortex and the spinal cord3–4. FTD is a group of neurodegenerative disorders that are characterized by neuronal loss in the frontal and temporal cortices5. ALS and FTD share common genetic causes, including mutations in TAR DNA-binding protein 43 (TDP-43, encoded by Tardbp)6,7. ALS and FTD also exhibit similar neuropathology with ubiquitinated protein inclusions containing TDP-43 (ref. 8), suggesting common pathological aspects of ALS and FTD via overexpression of mutant TAR DNA-binding protein 43 (TDP) mice, an ALS and FTD model with marked cortical pathology, we found that hyperactive somatostatin interneurons disinhibited layer 5 pyramidal neurons (L5-PNs) and contributed to their excitotoxicity. Focal ablation of somatostatin interneurons efficiently restored normal excitability of L5-PNs and alleviated neurodegeneration, suggesting a new therapeutic target for ALS and FTD.

To illustrate the potential disinhibition connectivity between Sst interneurons and L5-PNs in M1 cortex were sustained throughout disease progression in TDP mice (Supplementary Fig. 2 and Supplementary Table 2). To visualize excitotoxicity of L5-PNs in TDP mice, we examined dendritic morphological changes, somatic cellular pathologies and potential neurodegeneration of L5-PNs at different disease stages. We found that dendritic blebs (an early sign of excitotoxicity17) appeared at an early stage (Fig. 1c, Supplementary Fig. 3a–d and Supplementary Table 3), whereas ubiquitin-positive aggregates and substantial reduction of L5-PNs appeared at the late disease stage of TDP mice (Fig. 1d, Supplementary Fig. 3e and Supplementary Table 4). Together, our data suggest that sustained impairments in GABAergic transmission of L5-PNs lead to its hyperexcitability, excitotoxicity and neurodegeneration in TDP mice.

To determine whether cortical inhibition is impaired in TDP mice, we first recorded miniature inhibitory postsynaptic currents (mIPSCs) and evoked IPSCs (eIPSCs) from L5-PNs in M1 cortex of postnatal 3-week-old TDP mice and their disease non-carrier littermates (wild type, WT). We found that both mIPSCs and eIPSCs were greatly reduced in TDP mice (Fig. 1a and Supplementary Fig. 1a). In addition, GABAergic synapse densities around somatic areas of L5-PNs were greatly reduced in TDP mice (Supplementary Fig. 1b). In contrast, excitatory transmissions were similar in WT and TDP mice (Supplementary Fig. 1c).

L5-PNs from 3-week-old TDP mice exhibited hyperexcitability that was abolished by intracellular application of picrotoxin, a GABAA receptor blocker (Fig. 1b, Supplementary Fig. 1d,e and Supplementary Table 1). This observation suggests that hyperexcitability of L5-PNs primarily originates from reduced GABAergic transmissions in TDP mice. The abnormalities in GABAergic transmission and L5-PN hyperexcitability in M1 cortex were sustained throughout disease progression in TDP mice (Supplementary Fig. 2 and Supplementary Table 2).

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To directly test the hypothesis that sustained hyperactive Sst interneurons cause excitotoxicity of L5-PNs in TDP mice, we next asked whether eliminating Sst interneurons in early adulthood of TDP mice would restore normal excitability of L5-PNs and rescue cortical neuropathology and neurodegeneration (Supplementary Fig. 7). We genetically labeled Sst interneurons with diphtheria toxin receptor...
Figure 3 Bilateral ablation of Sst interneurons diminished excitotoxicity of L5-PNs in TDP mice. (a) 2-week bilateral ablation of Sst interneurons in M1 cortex of TDP mice increased L5-PN mIPSC frequency. Left, representative traces of L5-PN mIPSCs. Right, dot plots of the frequencies of mIPSCs (S, saline; groups 1–4, n = 25, 26, 27 and 28 neurons, 3 mice; one-way ANOVA and post hoc Tukey test). (b) 2-week bilateral ablation of Sst interneurons in M1 restored normal excitability of L5-PNs in TDP mice. Left, representative traces of AP firing of L5-PNs through loose-seal cell-attached recordings with bath application of 20 mM KCl. Right, dot plots of AP firing frequencies (groups 1–4, n = 15, 18, 12 and 10 neurons, 3, 4, 4 and 3 mice; Brown and Forsythe test and post hoc Games-Howell test). (c) 6-week bilateral ablation of Sst interneurons in M1 cortex increased vesicular GABA transporter (VGAT) puncta in L5-PNs. Left, representative images of VGAT-staining (red, VGAT; green, NeuN; scale bar represents 10 µm). Right, dot plots of puncta densities (groups 1–4, n = 180, 120, 143 and 195 neurons, 3, 2, 3 and 3 mice; Brown and Forsythe test and post hoc Games-Howell test). (d) 6-week bilateral ablation of Sst interneurons reduced ubiquitin-positive aggregates in TDP mice. Left, representative images (red arrows indicated ubiquitin-positive neurons; scale bar represents 100 µm). Right, dot plots of ubiquitin-positive neuron numbers (groups 1–4, n = 24, 24, 36 and 42 counts, from 12, 12, 18 and 21 slices of 4, 4, 6 and 7 mice; Mann-Whitney U test). (e) 6-week bilateral ablation of Sst interneurons reversed neuronal loss in M1 cortex of TDP mice. Left, representative images of NeuN immunostaining (scale bar represents 20 µm). Right, dot plots of layer 5 neuron densities (groups 1–4, n = 18, 16, 24 and 24 counts from 9, 8, 12 and 12 slices of 3, 3, 4 and 4 mice; Brown and Forsythe test and post hoc Games-Howell test). Data are presented as mean ± s.e.m. * P < 0.05; ** P < 0.01; *** P < 0.001; N.S., not significant (P > 0.05).

(DTR) and injected diphtheria toxin (DT) locally to bilaterally ablate Sst interneurons in M1 cortex in 6-week-old mice (Supplementary Fig. 8). We found a substantial increase in the frequency of mIPSCs of L5-PNs 2 weeks after Sst interneuron ablation in TDP mice (Fig. 3a). Loose-seal cell-attached recordings of L5-PNs revealed that Sst interneuron ablation in TDP mice fully restored the spiking activity of L5-PNs to levels similar to those of disease non-carrier controls (Fig. 3b). In separate groups of TDP mice following 6 weeks bilateral DT injection, we found that Sst interneuron ablation in TDP mice significantly increased GABAergic synaptic density on L5-PN, reduced ubiquitin-positive aggregates in M1 cortex and reversed the neuronal loss in M1 cortex of TDP mice (Fig. 3c–e). Together, these results support our hypothesis that the excitotoxicity of L5-PN is primarily driven by sustained hyperactive Sst interneurons in M1 cortex of TDP mice.

In sum, we discovered a specific microcircuit between Sst interneurons and L5-PNs in M1 cortex in which Sst interneurons send an overall disinhibitory signal to L5-PNs via inhibiting Pv interneurons. Notably, we found that, in TDP mice, this inhibitory neuronal circuit is dysfunctional and therefore contributes to the cortical pathogenesis in ALS and FTD. We propose targeting this subpopulation of inhibitory interneurons as a therapeutic concept for ALS and FTD.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.L., D.-T.L., W.Z. and L.Z. designed the study and wrote the manuscript. W.Z. and L.Z. performed all of the experiments and analysis. B.L. helped with data analysis. D.S. maintained some mouse colonies. G.A.C. and Z.Z. advised on experiments and manuscript preparation.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Mice.** All experiments were conducted in accordance with procedures established by the Administrative Panels on Laboratory Animal Care at The Rockefeller University and US National Institutes of Health. Transgenic mice used in this manuscript were: B6.Cg-Tg(PrpTARP-DARP:A1351T)95Bal/j (101700, TDP-43*117T* or TDP); B6.Cg-Tg (Thy1-YFPH2cre)1 (003782, Thy1-YFPH or YFP); B6.Cg-Gt(Rosa)26Sor1m1(CAG-tdTomato)HloxP/tdTomato (007914, tdTomato); STOCK Ssttm2.1(cre)Zjh (013044, SstCre or Sst); B6.Cg-PrbAltm1(cre)Adh (012358, PrbCre or Pv); C57BL/6-Gt(Rosa)26Sor1m1(HBGCre)HloxP/tdTomato (007900, DTR). Male mice were used across the studies.

**Slice preparation.** Male mice age P20-22, P50-60, and P90–100 were killed with CO2 and decapitated. 250-µm coronal slices were prepared with a vibratome (Leica VT1000 S and VT1200) in ice-cold cutting solution contained the following (in mM): 80 NaCl, 26 NaHCO3, 3.0 KCl, 1.0 NaH2PO4, 1.3 MgCl2, 1.0 CaCl2, 20 n-glucose, and 75 sucrose, saturated with 95% O2 and 5% CO2. Slices were then moved to an incubation chamber containing artificial cerebrospinal fluid (ACSF) contained the following (in mM): 124 NaCl, 26 NaHCO3, 3.0 KCl, 1.0 NaH2PO4, 1.3 MgCl2, 1.5 CaCl2, 20 glucose, saturated with 95% O2 and 5% CO2. Slices were incubated first at 34 °C for 30 min and then at 21 °C until used for recordings.

**Electrophysiological recordings.** L5-PN in M1 cortex was identified by cell morphology and size, and recorded using methods as previously described. Brain slices were placed in a submersion type chamber continuously perfused with ACSF saturated with 95% O2 and 5% CO2 at 23–25 °C until used for recordings. For current-clamp recording, the pipette solution contained (in mM): 120 potassium gluconate, 10 KCl, 4 ATP-Mg, 0.3 GTP , 0.5 EGTA, 10 HEPES, and 0.5 EGTA (pH 7.2, 300–320 mOsm with sucrose). Miniature IPSC recordings were performed in the presence of 20 µM DNXQ, 50 µM AFS, and 1 µM tetrodotoxin (TTX). For recordings of evoked IPSC, a concentric bipolar electrode (FHC) was placed in L5 of M1 cortex at a fixed distance of 200 µm to the recorded cell to apply stimulus.

**For EPSC recordings, the pipette solution contained (in mM): 110 cesium methylsulfate, 15 CsCl, 4 ATP-Mg, 0.3 GTP, 0.5 EGTA, 10 HEPES, and 4.0 QX-314 (pH 7.2, 270–280 mOsm with sucrose). Miniature EPSC (mEPSC) was recorded at a distance of 1 µM TTX and 100 µM picrotoxin.**

Electrodes had resistances between 2 and 3.5 MΩ. The series resistance was not compensated in voltage-clamp experiments. During experiments, the series resistance was constantly monitored. Data were discarded when series resistance was >16 MΩ or change of series resistance was >15%.

**For current-clamp recording, the pipette solution contained (in mM): 120 potassium gluconate, 10 KCl, 4 ATP-Mg, 0.3 GTP, 0.5 EGTA, and 0.5 EGTA (pH 7.2, 270–280 mOsm with sucrose).** Series resistance was fully compensated using the bridge circuit of the amplifier Multiclamp 700B. Action potential threshold was estimated as the point when the slope of rising membrane potential exceeds 50 mV ms−1.

For photo-stimulation of ChR2 and eNpHR3.0, we used a high-power light source (HXP 120V, Zeiss) controlled by AxoGraph X with TTL signal. Light was delivered onto brain slices through a band-pass filter (470–740 nm for ChR2, 605–670 nm for eNpHR30) via a 40 X objective (NA 0.8).

For loose-seal cell-attached recordings of L5-PN, the electrodes were filled with ACSF and a loose patch of >50 MΩ was achieved for recording with the bath application of 20 mM KCl. Recordings were performed with Multiclamp 700B (Molecular Devices). Experiments were conducted using Axograph X and Igor Pro (Wavemetrics). Synaptic systems were analyzed offline using Axograph X and Igor Pro (Wavemetrics). For electrophysiological recordings, we used custom two-photon microscope operated using custom software (ScanImage). Fine vascular patterns observed through the cranial window were used as landmarks to enable repeated imaging of the same group of neurons. We obtained the initial in vivo two-photon images of L5-PN two days after the surgery day. At the age of 9-weeks or 15-weeks we re-thinned the original cranial window and obtained the repetitive images.

**Morphology and size are the two main criteria to identify ‘blebs’. The morphology of blebs is unique as ‘beads on a string’, which is very different from regular dendritic spine. We initially identified ‘blebs’ manually based on the morphology.** And the size of L5-PN dendritic blebings was quantified, and the number of dendritic blebings was quantified and normalized with corresponding image areas across groups. We found that the size of blebs was much bigger than that of spines. A spine is in general less than 1 µm² (area), while a bleb is in an average of ~12.8 µm² (ranging from 2.7 µm² to 64 µm²). We also counted blebs ‘semi-automatically’: first set up a threshold of 3 µm² to automatically identify potential blebs; then manually exclude the false counts resulting from dendritic intersections. These two methods gave us similar results. We only presented the data analysis from manual identification.

**Immunostaining.** Mice were anesthetized with ketamine and xylazine (100 mg kg⁻¹ and 10 mg kg⁻¹, respectively). For VGAT and P and staining, mice were perfused with phosphate-buffer saline (PBS, pH 7.4) and then 4% paraformaldehyde (PFA) in PBS. Brain tissues were post-fixed with 4% PFA in PBS overnight at 4 °C, and 40-µm coronal sections were prepared with vibratome. VGAT and P and immunostaining followed the standard protocols for free-floating sections. In brief, free-floating sections were incubated in blocking solution containing 4% normal goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS, with slowly shaking for 2 h at 23–25 °C. Sections were then treated with primary antibody in blocking solution overnight at 4 °C and with secondary antibody in blocking solution at 23–25 °C for 2 h with slowly shaking. Stacked images (4 µm each stack) were obtained through Leica Confocal Microscopy.

For ubiquitin staining, mice were perfused with PBS and then Bouin’s solution. Brain tissues were post-fixed in Bouin’s solution overnight at 4 °C and submitted blindly to the Jackson Laboratory Histology Core to be processed for paraffin embedding and ubiquitin staining. Immunostaining with paraffin embedded sections was similar procedure except that the sections were deparaffinized and went through the antigen retrieval step (microwave boiling for 9 min in 0.01 M citrate, pH = 6.0). Primary antibodies used were mouse anti-parvalbumin (1:1,000 dilution, p3088, Sigma-Aldrich), rabbit anti-VGAT (1:2,000 dilution, 131003, Synaptic Systems), rabbit anti-somatostatin (1:300 dilution, sc-13099, Santa Cruz Biotechnology), mouse anti-NeuN (1:1,000 dilution, ab104224, Abcam) and mouse anti-ubiquitin (1:500 dilution, 3936, Cell Signaling Technology). Secondary antibodies were used Alexa Fluor 647 donkey anti-mouse IgG (1:300 dilution, 715-605-150, Jackson ImmunoResearch Laboratories) and Alexa Fluor 647 donkey anti-rabbit IgG (1:300 dilution, 711-605-152, Jackson ImmunoResearch Laboratories).

*Image* software was used for VGAT puncta density analysis from brain slices of YFP and TDP-43:YFP mice with VGAT immunostaining. YFP signal was used to outline somatic area of L5-PN. VGAT puncta inside the somatic outline were quantified through Particle Analysis with parameters of 0.1–4 µm in size and 0.1–1.00 in circularity. Custom script in MATLAB was used to calculate the density of VGAT puncta from brain slices of VGAT and NeuN co-immunostaining.

Briefly, VGAT signals with intensity three times above the s.d. of the image background were detected. The NeuN signal was used to define the region of interest (ROI). Only ROIs within layer 5 and with a minimal area of 300 µm² were identified as presumably L5-PN and pursued for further analysis. VGAT puncta for each identified ROI was quantified with a spot-detection algorithm.

For NeuN-positive cell count, images of layer 5 in M1 cortex were taken with a confocal microscope (Zeiss LSM 710) equipped with a 40× objective (NA 1.3),
then NeuN-positive cell density was calculated by dividing cell number by the image area (212.55 µm × 212.55 µm).

**Injection of AAV virus for ChR2, eNpHR3.0, or eYFP expression.** Adeno-associated viruses (AAV1) for Cre dependent expression of ChR2, eNpHR3.0, and eYFP were acquired from the University of Pennsylvania Viral Vector Core: pAAV1-EF1a-DIO-hChR2(H134R)-eYFP-WPRE-pA (titer 3.4 × 10^{12} genome copies each milliliter), pAAV1-EF1a-DIO-eNpHR3.0-eYFP-WPRE-pA (titer 7 × 10^{12} genome copies each milliliter), and pAAV1-EF1a-DIO-eYFP-WPREehGH (titer 1.8 × 10^{13} genome copies each milliliter). To perform optogenetic experiments, Cre dependent ChR2 or eNpHR3.0 viruses were 1:5 diluted with saline and injected into the M1 cortex of postnatal 6–7 weeks of virus were 1:5 diluted with saline and injected into the M1 cortex of postnatal 6–7 weeks of TDP mice. To identify Sst and Pv interneurons in adulthood TDP mice, Cre dependent eYFP (titer 1.8 × 10^{13} genome copies each milliliter). To perform optogenetic experiments, Cre dependent expression of ChR2, eNpHR3.0, and eYFP virus injection mice were killed for electrophysiology experiments.

**Toxin injection.** 6-weeks old mice were anesthetized with 2–2.5% isoflurane for diphtheria toxin (DT) injection, placed on a heating pad maintained at 37 °C. 0.5 µl of DT (100 pg µl−1 in saline) was stereotaxically injected bilaterally into the M1 cortex region (AP: +1.5 mm, ML: +2.0 mm) of postnatal 6–7 weeks of PvCre and SstCre mice at a depth of 1.25 mm using a micropump (WPI). A total of 300 nanoliter of virus was injected over 5 min. To identify Sst and Pv interneurons in adulthood TDP mice, Cre dependent eYFP virus were 1:5 diluted with saline and injected into the M1 cortex of postnatal 6–7 weeks of PvCre, TDP-43::PvCre, SstCre, and TDP-43::SstCre mice. 2 weeks after virus injection mice were killed for electrophysiology experiments.

**Sample size, randomization and blinding statement.** Sample sizes for electrophysiological recordings and for in vivo imaging and immunostainings were estimated based on past experience and those presented in the literature. Typically, recordings of n > 10 neurons from at least three mice each group were collected for electrophysiological studies; n > 10 images from at least five mice each group were collected for in vivo imaging; and n > 10 counts from each side of slices from at least three mice each group were collected for immunostaining. Mice were randomly allocated to treatment condition and all data were randomly collected. Initial electrophysiological recordings (that is, mIPSCs), ubiquitin stainings, NeuN immunostainings and VGAT immunostainings were performed in a blinded manner. All other data were collected and analyzed without the investigator blinded to genotype and treatment conditions.

**Statistical analysis.** All statistical comparisons were performed with two-sided tests. For comparisons between two groups, D'Agostino & Pearson omnibus test was used for normality test and the variances were calculated with Prism5.0 analysis function. If both groups displayed normal distributions and had equal variance, unpaired t test was used; otherwise non-parametric Mann-Whitney U test was used. For comparisons between multiple groups, Jarque-Bera test was used for normality test, and Levene's test was used for variance test. If all groups have normal distribution and equal variance, one-way ANOVA with post hoc Tukey test was used. If multiple groups exhibited different variances, Brown and Forsythe Test with post-hoc Games-Howell test were used. In these tests, data distribution was assumed to be normal but this was not formally tested. For experiment with photoactivation of ChR2 and eNpHR3.0, Wilcoxon signed rank test and paired t test were used (Jarque-Bera test was used for normality test, and Levene's test was used for variance test). P < 0.05 was accepted as statistically significant.

**Code availability.** Custom script in MATLAB, which was used for VGAT puncta density analysis, is available on request.

A Supplementary Methods Checklist is available.

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