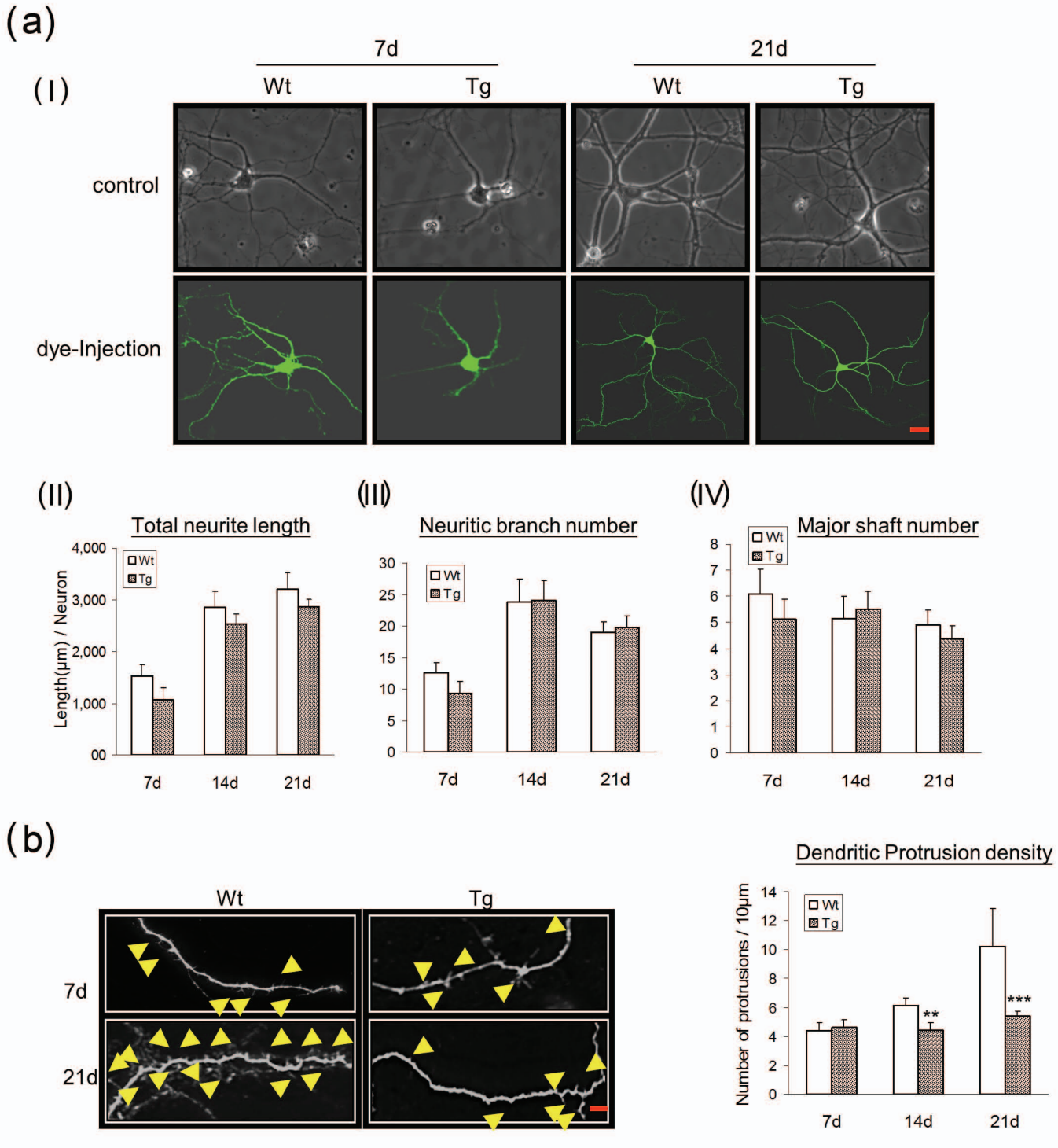
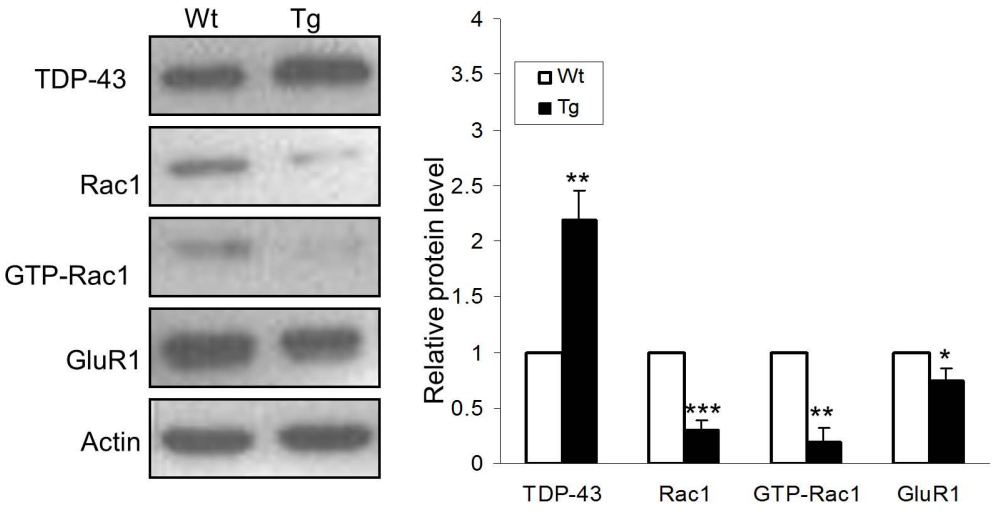
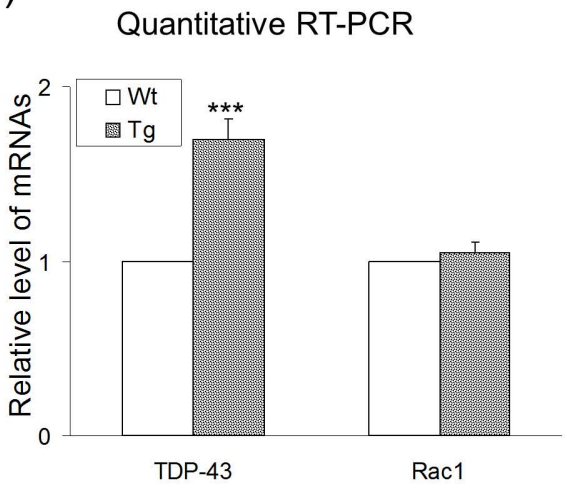

(a)
(I)
7d
21d
Wt
Tg
Wt
Tg
control
dye-Injection
(II)
Total neurite length
Length(μm) / Neuron
4,000
3,000
2,000
1,000
00
Wt
Tg
7d
14d
21d
(III)
Neuritic branch number
30
25
20
15
10
5
0
Wt
Tg
7d
14d
21d
(IV)
Major shaft number
8
7
6
5
4
3
2
1
0
Wt
Tg
7d
14d
21d
(b)
Wt
Tg
7d
21d
Dendritic Protrusion density
Number of protrusions / 10μm
14
12
10
8
6
4
2
0
Wt
Tg
**
***
7d
14d
21d

(a)

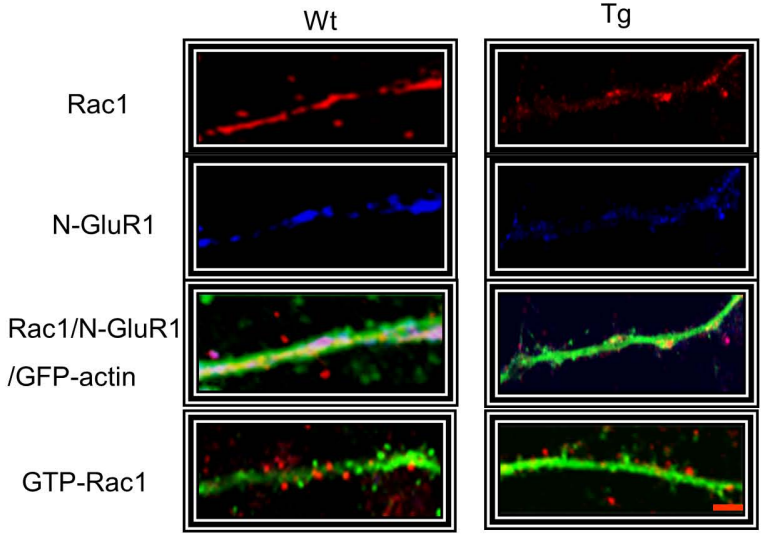
(I)

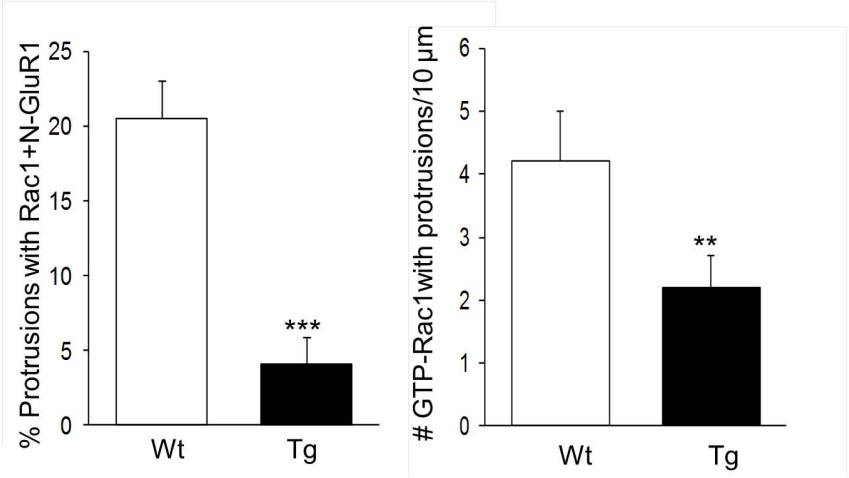

Wt
Tg
TDP-43
Rac1
GTP-Rac1
GluR1
Actin
Relative protein level
Wt
Tg
TDP-43
Rac1
GTP-Rac1
GluR1

(II)

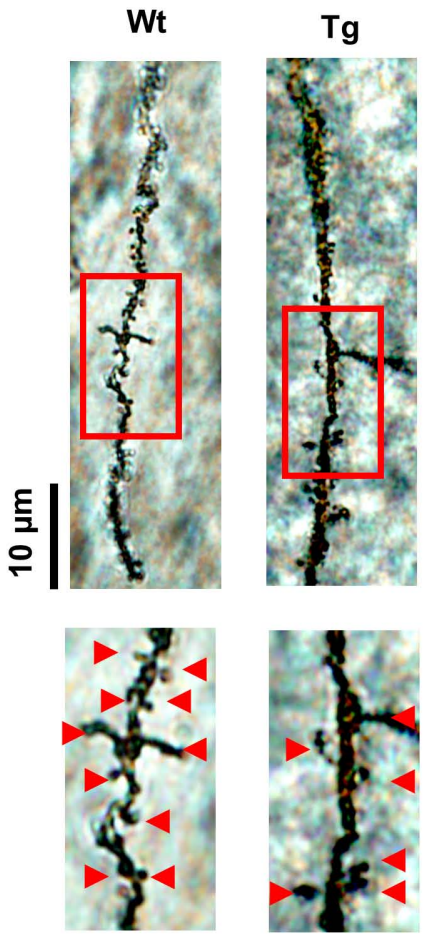

Quantitative RT-PCR
Relative level of mRNAs
Wt
Tg
TDP-43
Rac1

(b)

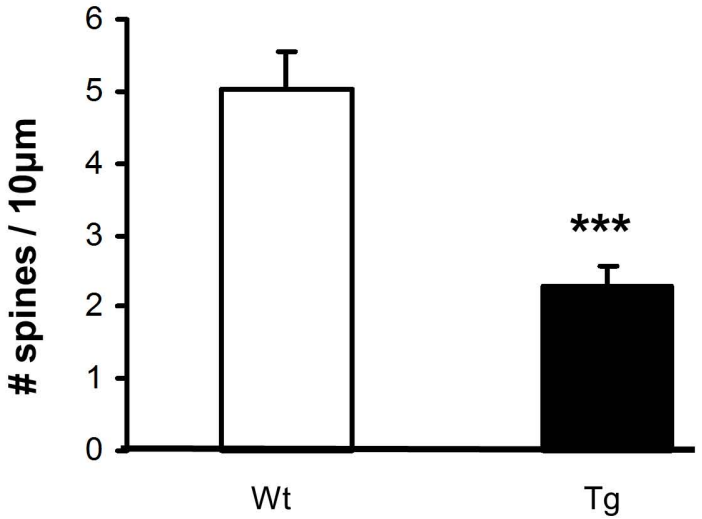

Wt
Tg
Rac1
N-GluR1
Rac1/N-GluR1
/GFP-actin
GTP-Rac1


% Protrusions with Rac1+N-GluR1
Wt
Tg
GTP-Rac1with protrusions/10 µm
Wt
Tg

(a)

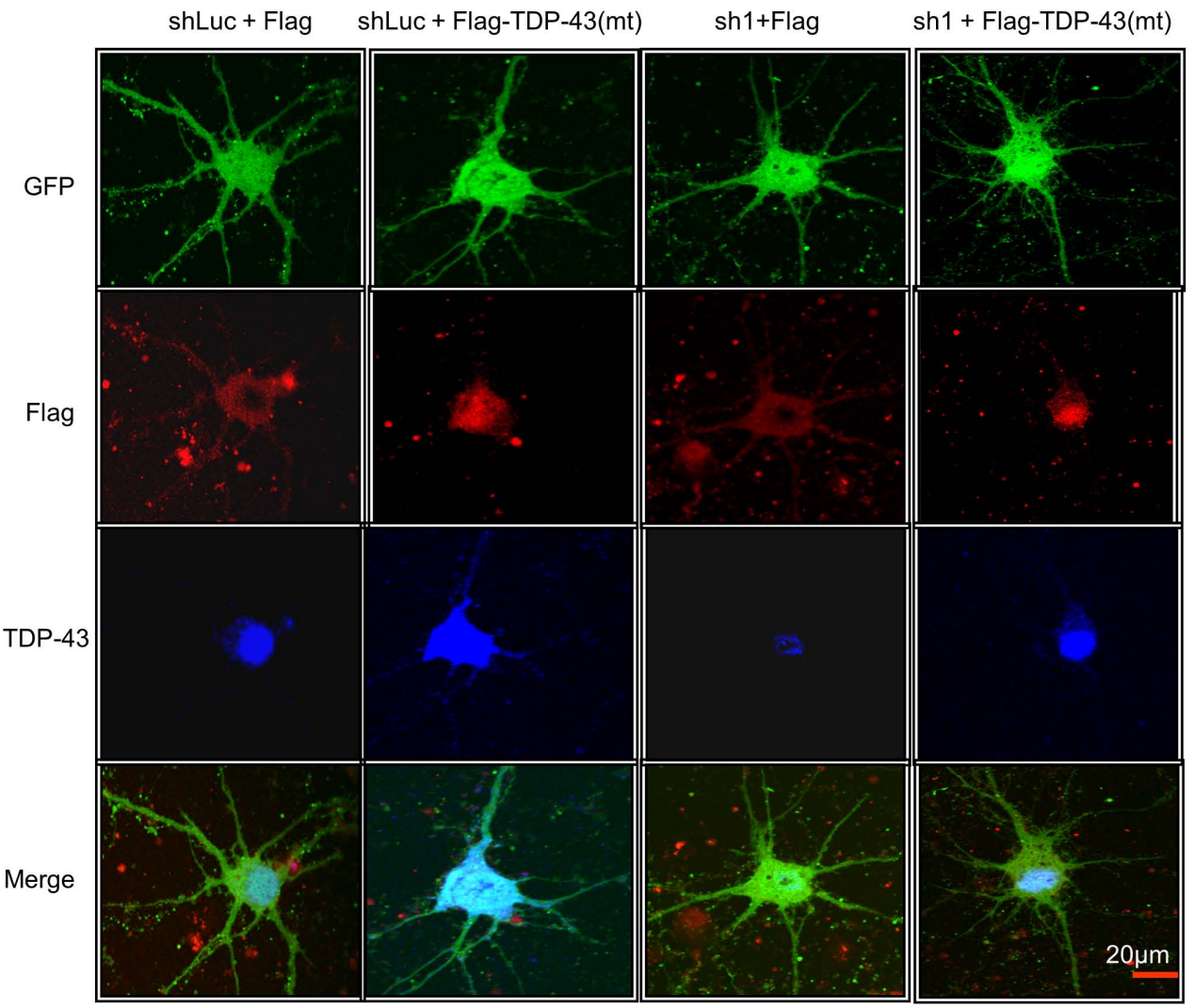


(b)

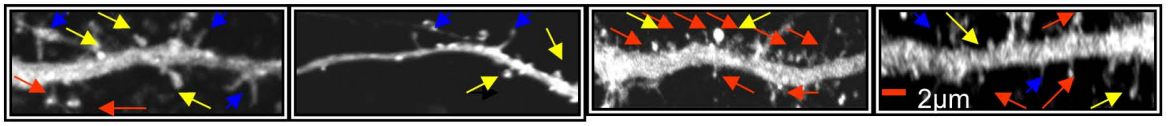


(a)

(I)

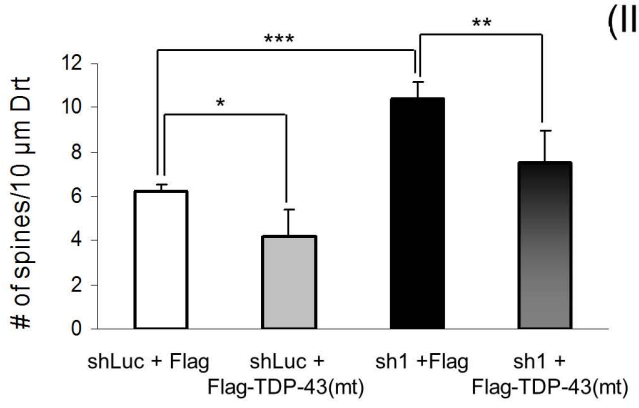

shLuc + Flag
shLuc + Flag-TDP-43(mt)
sh1+Flag
sh1 + Flag-TDP-43(mt)
GFP
Flag
TDP-43
Merge
20μm

(II)

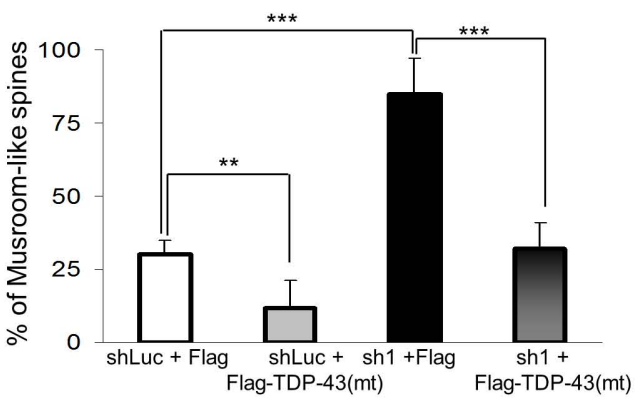

2μm

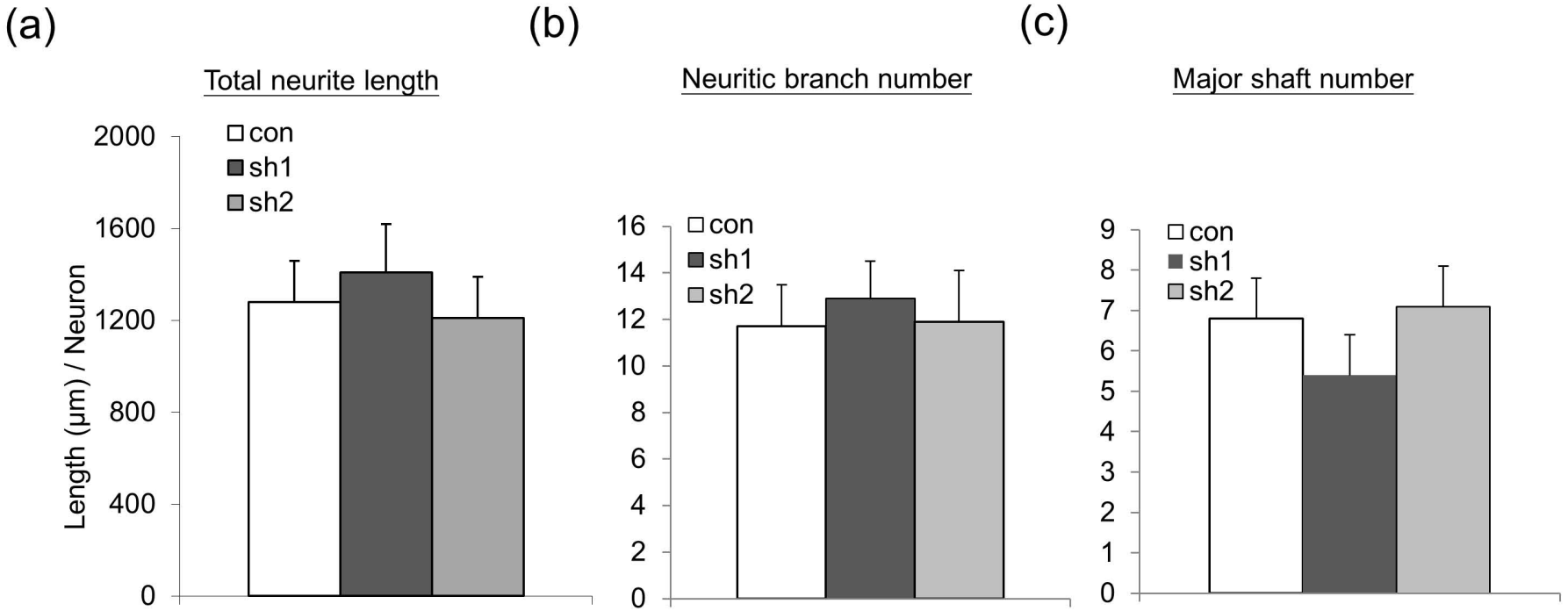
(b)

(I)

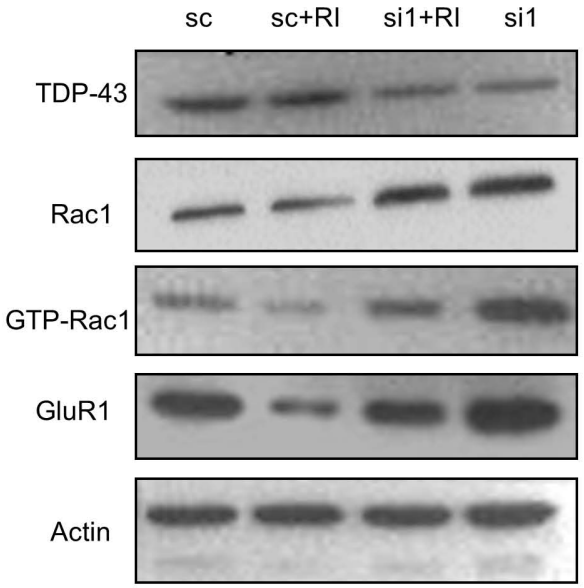

# of spines/10 μm Drt
12
10
8
6
4
2
0
***
**
*
shLuc + Flag
shLuc + Flag-TDP-43(mt)
sh1 +Flag
sh1 + Flag-TDP-43(mt)

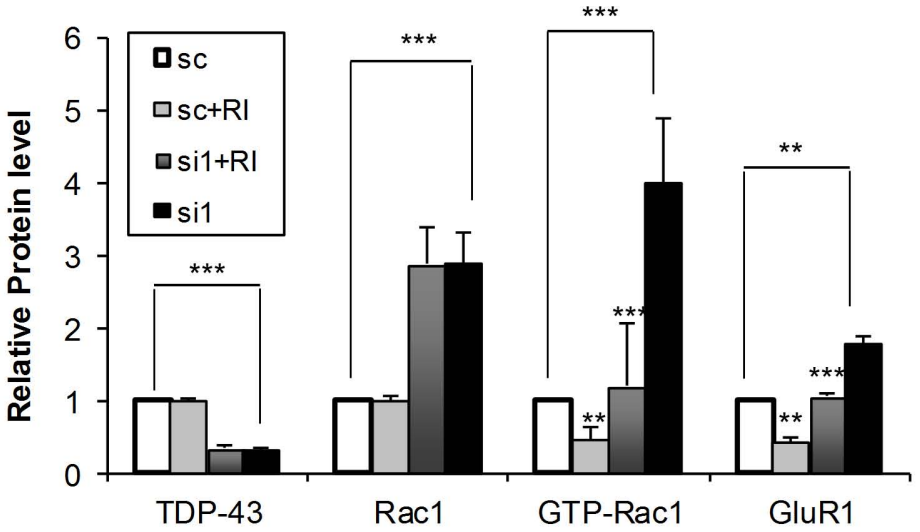
(II)


% of Musroom-like spines
100
75
50
25
0
***
***
**
shLuc + Flag
shLuc + Flag-TDP-43(mt)
sh1 +Flag
sh1 + Flag-TDP-43(mt)

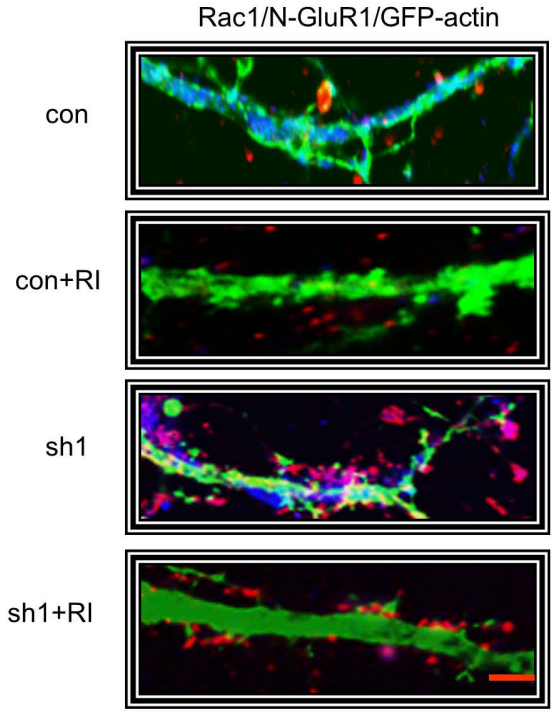

(a)
Total neurite length
Length (μm) / Neuron
2000
1600
1200
800
400
0
con
sh1
sh2
(b)
Neuritic branch number
16
14
12
10
8
6
4
2
0
con
sh1
sh2
(c)
Major shaft number
9
8
7
6
5
4
3
2
1
0
con
sh1
sh2

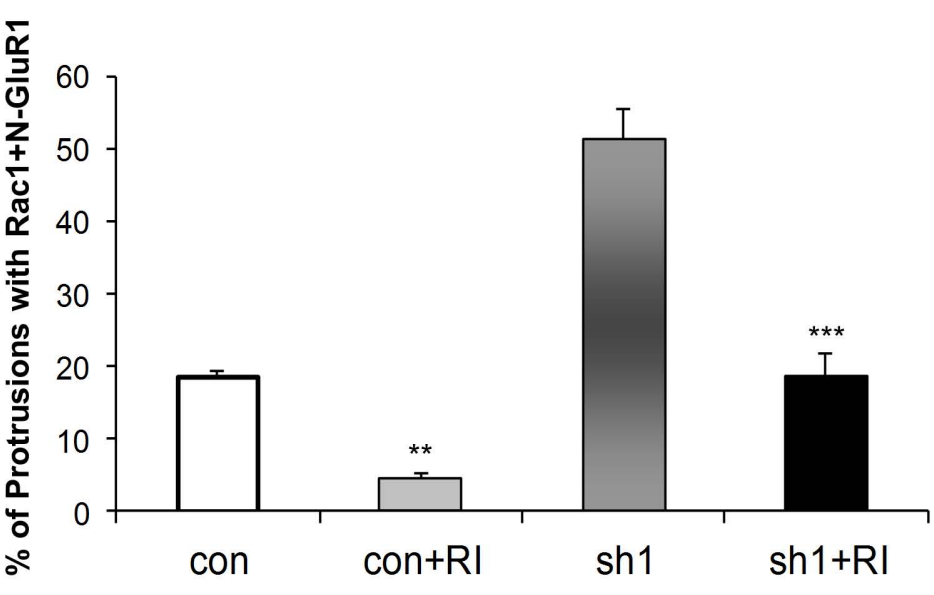
(a)


sc
sc+RI
si1+RI
si1
TDP-43
Rac1
GTP-Rac1
GluR1
Actin

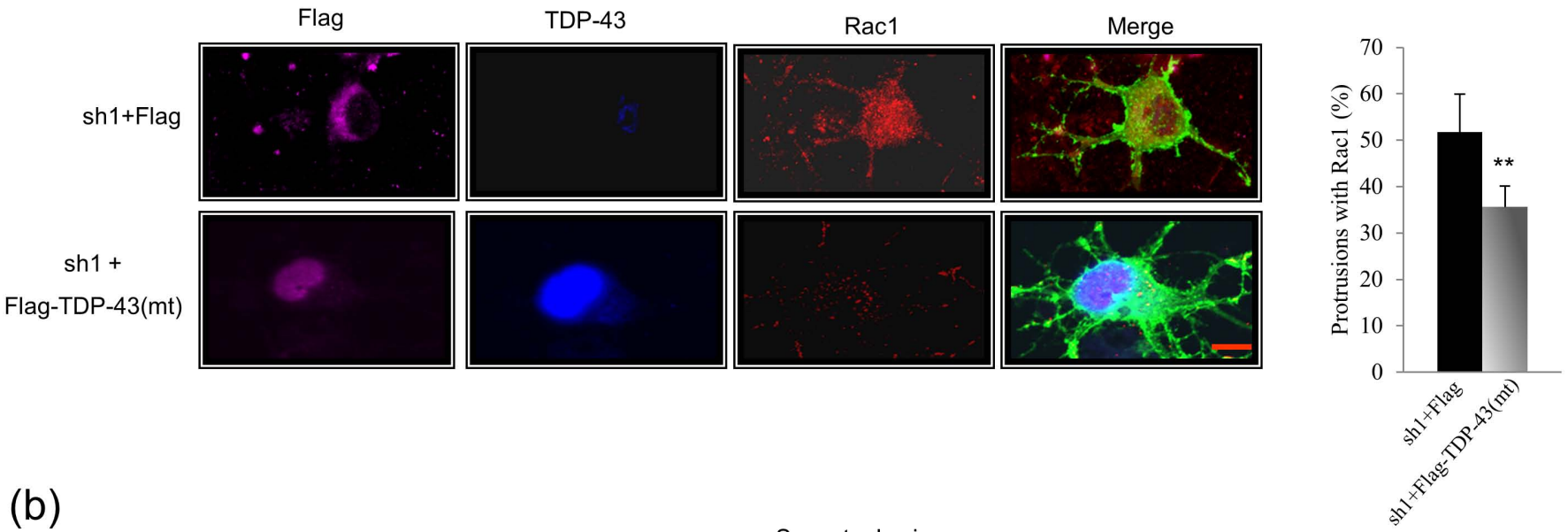

Relative Protein level
sc
sc+RI
si1+RI
si1
TDP-43
Rac1
GTP-Rac1
GluR1

(b)

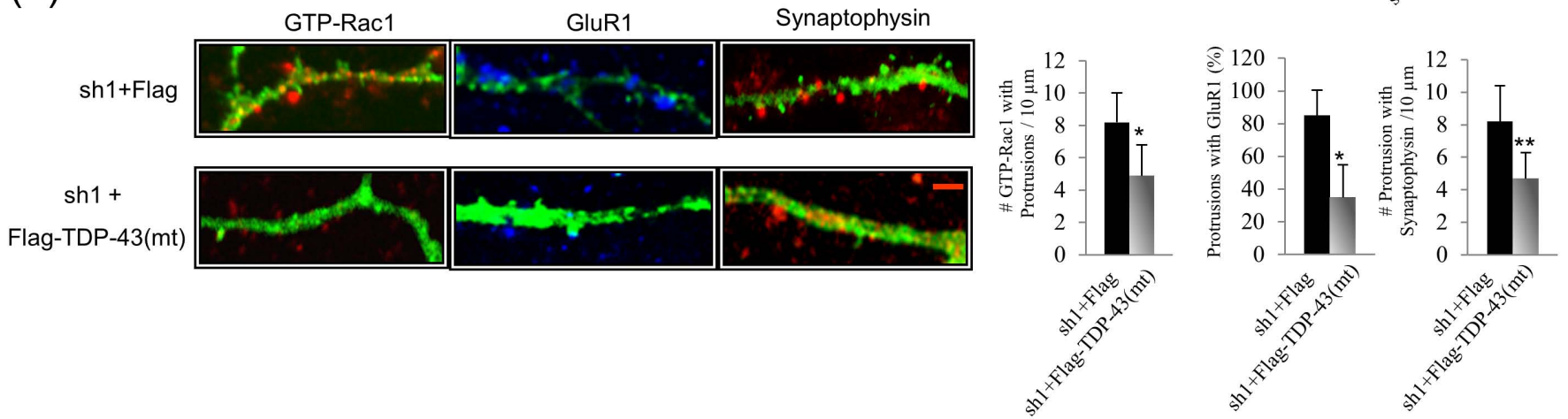

Rac1/N-GluR1/GFP-actin
con
con+RI
sh1
sh1+RI


% of Protrusions with Rac1+N-GluR1
con
con+RI
sh1
sh1+RI

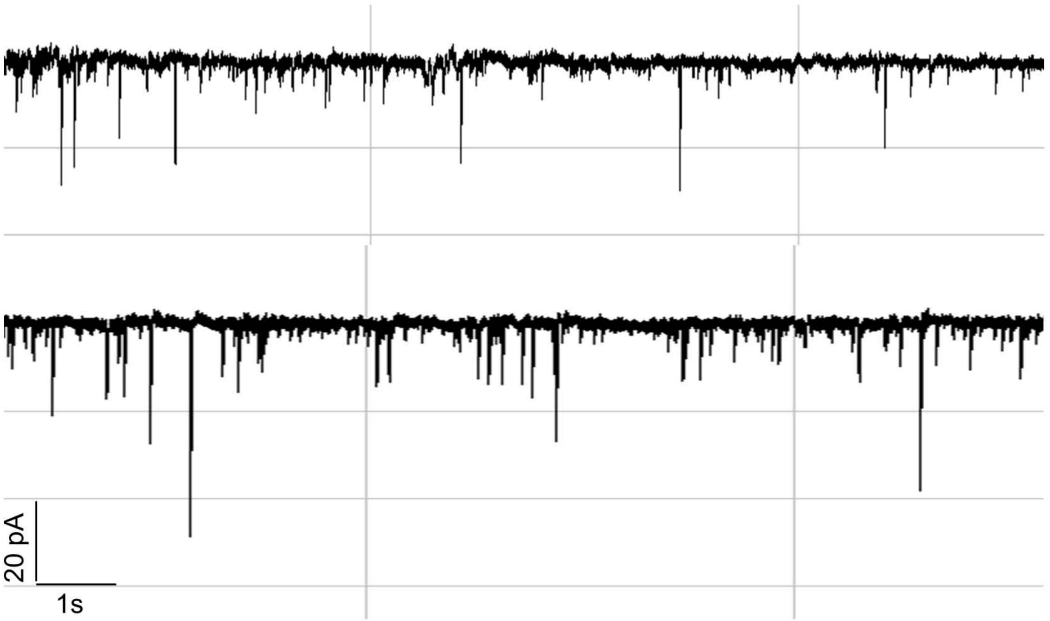
(a)


Flag
TDP-43
Rac1
Merge
sh1+Flag
sh1 +
Flag-TDP-43(mt)
Protrusions with Rac1 (%)
70
60
50
40
30
20
10
0
**
sh1+Flag
sh1+Flag-TDP-43(mt)

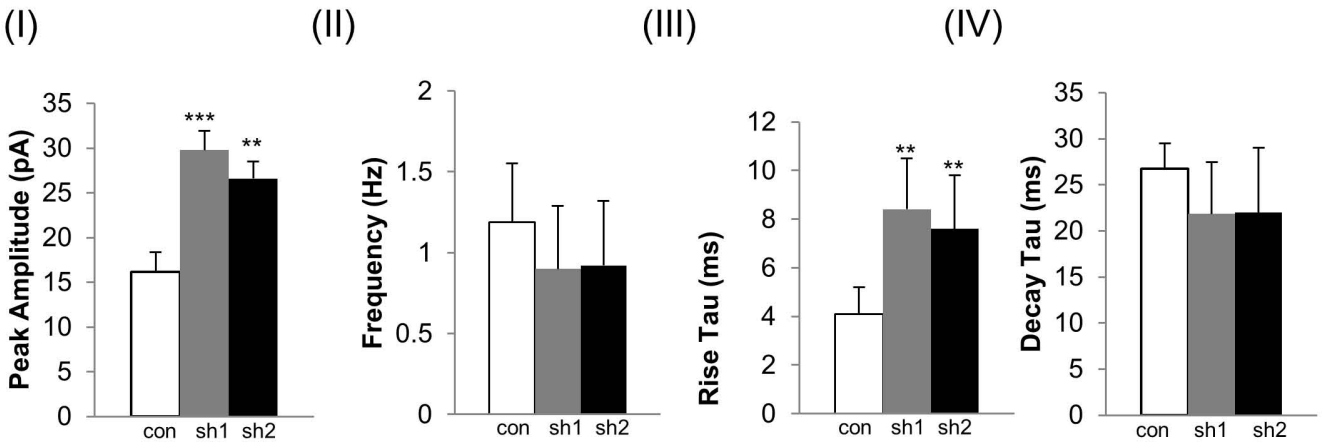
(b)


GTP-Rac1
GluR1
Synaptophysin
sh1+Flag
sh1 +
Flag-TDP-43(mt)
GTP-Rac1 with Protrusions / 10 µm
12
10
8
6
4
2
0
*
sh1+Flag
sh1+Flag-TDP-43(mt)
Protrusions with GluR1 (%)
120
100
80
60
40
20
0
*
sh1+Flag
sh1+Flag-TDP-43(mt)
Protrusion with Synaptophysin /10 µm
12
10
8
6
4
2
0
**
sh1+Flag
sh1+Flag-TDP-43(mt)

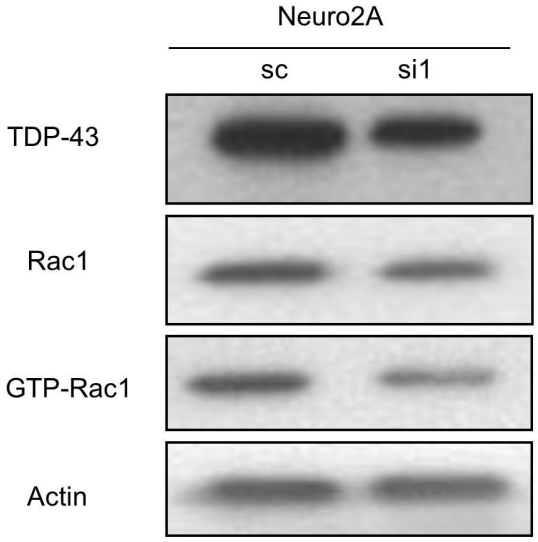
(a)


Control
sh1
20 pA
1s

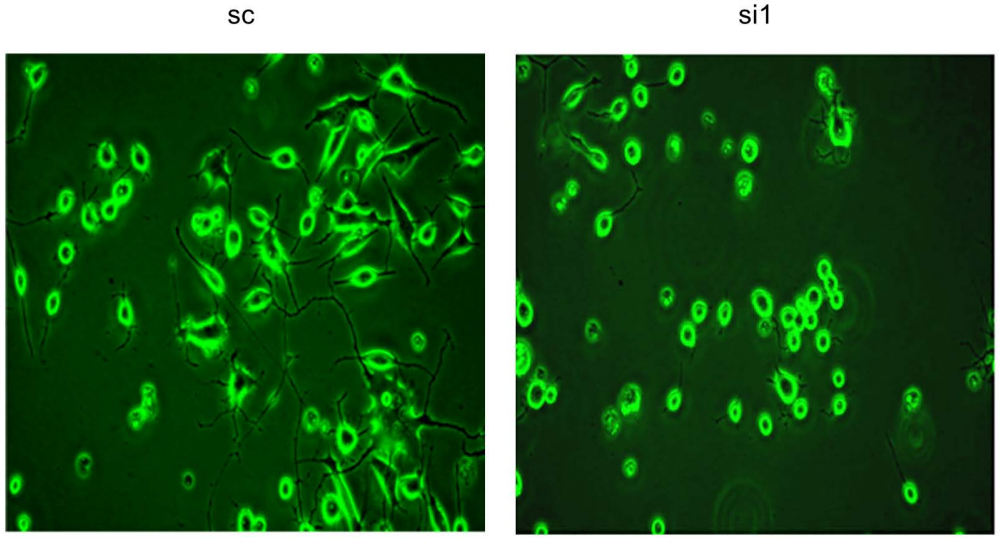
(b)


(I)
(II)
(III)
(IV)
Peak Amplitude (pA)
Frequency (Hz)
Rise Tau (ms)
Decay Tau (ms)
con
sh1
sh2
***
**

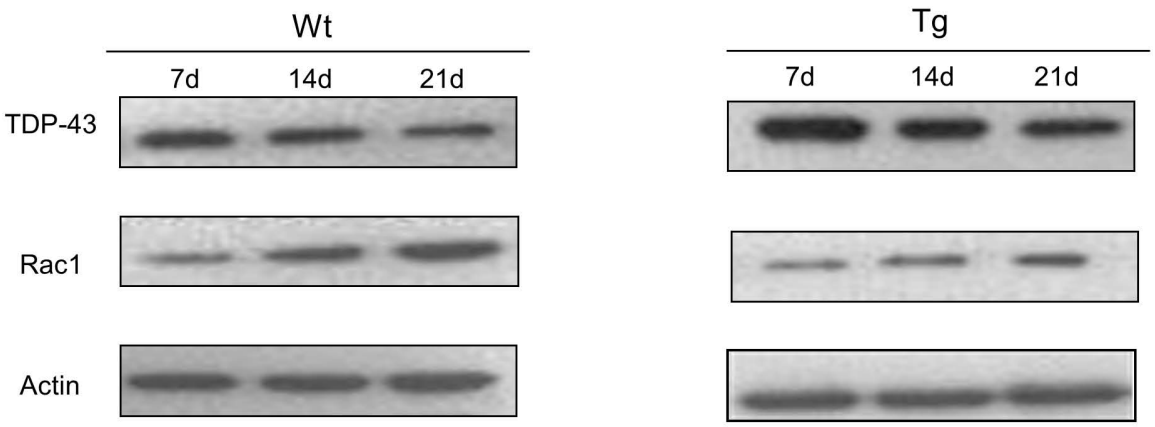
(a)



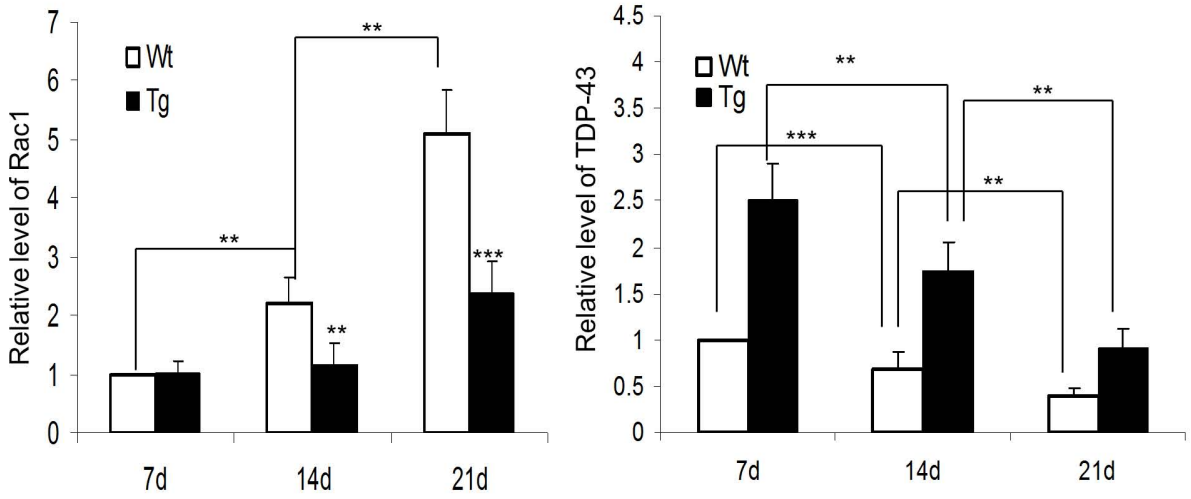
(b)



(a)


Wt
Tg
7d
14d
21d
7d
14d
21d
TDP-43
Rac1
Actin

(b)


Relative level of Rac1
Wt
Tg
**
**
**
***
0
1
2
3
4
5
6
7
7d
14d
21d
Relative level of TDP-43
Wt
Tg
**
***
**
**
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
7d
14d
21d

Supplementary Material

Title: TDP-43 Regulates The Mammalian Spinogenesis Through Translational Repression of Rac1

Running Title: Regulation of Spinogenesis by TDP-43

Authors:

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Supplimentary Materials and Methods

Mice and primary neuronal cultures

$GFP^{+/-}$ Tg mice (Jackson Laboratory, USA) were crossed with Wt and CamKII-TDP-43 Tg mice, respectively, to generate GFP-expressing Wt mice and GFP-expressing TDP-43 Tg mice. The preparation of cultured hippocampal neurons followed the standard protocols [8]. Briefly, the cells were mechanically dissociated from hippocampi of E16.5~17.5 mouse embryos. 2×10^5 cells in 2 ml of the neurobasal medium (Invitrogen) supplemented with B-27 were plated in each well of the 6-well plates containing poly-D-lysine (Sigma)-coated cover slips. After incubation for 2–3 hr at 37 ℃ with 5% CO_2, the culture medium was

changed. After another 4–7 days, half of the medium from each well was replaced with fresh neuronal culture medium.

Microinjection of single neurons

Primary hippocampal neurons grown on the cover slips and cultured for different days *in vitro* were transferred from the 6-well plates onto 35-mm culture dishes each containing 2 ml of pre-warmed (37 ℃) physiological extracellular solution, or the artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 25 mM $NaHCO_3$, 2.5mM KCl, 1.25mM NaH_2PO_4, 2mM $CaCl_2$, 1mM $MgCl_2$ and 25mM glucose. The microinjection pipettes used were made of sterile, unpolished borosilicate glass capillary (Sutter Instrument). The needles were loaded with 100 μM sulforhodamine 101, 0.2% of biocytin (Invitrogen) dissolved in 120 mM K-gluconate, 20 mM KCl, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM Na_2ATP and 10 mM HEPES, pH 7.3 [6.7]. Injection with slight swellings of the cell body afterwards was considered successful. The biocytin injection was followed by staining with Avidin-conjugated Alex488 (Invitrogen) to visualize the intact morphology of the single neuron(s) (see below).

Plasmid construction

pGFP-actin was from Clontech. p-sh1(TDP-43), p-sh2(TDP-43) and p-shLuc expressed the three different vector based RNAi that were capable of knocking down the mouse TDP-43 mRNA (sh1 and sh2) and luciferase mRNA (shLuc), respectively. Sequences of sh1 and sh2 are 5'-CCGGGTAGATGACTTCATTCCCAAACTCGAGTTTGGGAATGAAGACATCTACTTTTTTG-3' and '5-CCGGGCTTTGTTCGATTTACAGAATCTCGAGATTCTGTAAATCGAACAAAGCTTTTTTG-3', respectively. All three plasmids were acquired from the NSC RNAi Core at the Institute of Molecular Biology, Academia Sinica and they were based on the vector pLKO.1 containing the U6 promoter and a puromycin-resistance gene. pFlag-TDP-43 encoded Flag-tagged TDP-43 under the control of the EF promoter [1]. For rescue experiments, two silent mutations were introduced at nucleotide positions 660 and 672 of the wild type TDP-43 cDNA using the following primer sets: 5'-GATGTGTTCATTCCCA AGCCAT-3'; 5'-ATGGCTTGGGAATGAACACATC-3' and 5'-AAGCTTATGTCTGAATATATTCGG GTAAC-3'; 5'-GCGGCCGCCTACATTCCCCAGCCAGAAGAC-3'. The mutated TDP-43 cDNA was cloned in the pEF-Flag vector mentioned above, resulting in the plasmid pFlag-TDP-43(mt). The Flag-TDP-43 (mt) mRNA expressed from this plasmid was resistant to degradation by sh1 (data not shown).

RNAi knockdown of TDP-43 in Neuro2A cells and effect of TDP-43 depletion on neurite outgrowth

Neuro 2A cells in culture was transfected with siRNA-1 oligo (2ρm) or a control scRNA oligo for 48 hours. Western blot analysis was done with the protein isolated from sc or si1 transfected cells against TDP-43, Rac1 and GTP-Rac1 antibodies. A different set of TDP-43 depleted (si1) or control (sc) cells were subjected to RA treatment as described previously [5] and cells were checked for neurite outgrowth.

Immunocytochemistry

For immunofluorescence staining experiments, the primary hippocampal neurons grown on poly-L-lysine coated cover slips were washed with PBS and fixed using 4% PFA at DIV 7, 14, or 21. After permeabilization with 0.5% Triton X 100 in PBS, the neurons were blocked with 10% FBS (Jackson Immuno Research Laboratories) and then probed with different antibodies: anti-Rac1 (Millipore, 1:100), anti-PSD-95(Sigma, 1:300), anti-Synaptophysin (Sigma, 1:250), anti-GTP-Rac1 (New East Bioscience, 1:200), anti-GluR1 (Santa Cruz Biotech., 1:50), anti-Flag (Santa Cruz Biotech., 1:100) and anti-TDP-43 (GeneTex, 1:450), diluted in the blocking buffer at 4℃ overnight. After several washes, the cells were incubated with AlexaFluor 533 or AlexaFluor 564, AlexaFluor 647, and/or AlexaFluor 350 conjugated secondary antibodies (Invitrogen) diluted in the blocking buffer for 1 hr. The processed cells on the cover slips were then analyzed under LSM 510 meta two-photon microscope or LSM 710 microscope.

To detect the surface GluR1 (N-GluR1) and Rac1, the cultured neurons were immunostained with anti-N-GluR1 (CalBiochem, 1:50) and anti-Rac1 antibodies diluted in PBS without the prior permeabilization step. Also, none of the buffers used in this staining experiment contained Tween 20 or any other detergent.

Quantitative analysis of the morphologies of the neurons in the hippocampal culture

The pyramidal neurons in the hippocampal cultures were chosen for analysis based on two criteria. First, the cells had strong fluorescence intensity for observation of the structures of the spines/ protrusions clearly. Second, there was minimal or no-overlapping of the neurons with neighboring GFP-expressing cells. 6-10 neurons of each sample were scored and analyzed by confocal microscopy. The means of the individual parameters and the standard errors were calculated.

Using manual tracing and the 3D capabilities of the software, the following parameters were quantified for each neuron: (i) The total neurite length, which represented the summed length of all the neurite processes; (ii) The neuritic main shaft number, which represented the number of processes emerging from the soma; (iii) neuritic branch number representing the total number of the neurites segments; (iv) The dendritic density of the protrusions or spines, represented as the average number of the protrusions or spines per 10 μm of the neurites [2, 3]. Only the dendrites, but not axons, were chosen for this analysis and 2-3 dendrites were measured per neuron cell. The values obtained are presented as mean ± SEM. Student's *t*-test was applied to the group comparisons of each of the parameters. The differences were considered statistically significant with the p Values < 0.05.

Electrophysiological recordings

Whole-cell voltage clamp recordings were performed on the cultured hippocampal neurons using an Axopatch 200B amplifier (Molecular Devices). Miniature EPSCs (mEPSCs) were recorded from cultured hippocampal neurons that had been transfected with plasmids encoding GFP-tagged molecules. Attempts were made to patch the first transfected neuron that was found. If the establishment of whole-cell configuration was not successful, a second transfected neuron was attempted. No more than two neurons

were recorded from the same culture dish. For the miniature post-synaptic current (mEPSC) measurements, the cultured neurons were re-suspended in artificial cerebrospinal fluid (ACSF) with 200 µM 2-amino-5-phosphonopentanoic acid (NMDA receptor blocker; Sigma), 1 µM tetrodotoxin (TTX; sodium channel blocker; Tocris Bioscinces), and 100 µM picrotoxin (GABA receptor blocker; Sigma). Only cells that had a resting membrane potential less than -50 mV, stable capacitance, and resistance throughout the experiment were considered. The data recorded were digitized with Digidata 1322A (Molecular Devices) and analyzed with Clampfit 10.2 (Molecular Devices). Detection criteria for mEPSCs included peak amplitudes >3 pA, a fast rise time and a slow decay time.

Western Blotting and RT-PCR analysis

For Western blotting, the total proteins were isolated from the cultured hippocampal neurons using lysis buffer containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1% Protease Inhibitor Cocktail. 20 µg of the total proteins for each sample were separated by 10% SDS-PAGE and analyzed by Western blotting following the standard procedures. The antibodies used were anti-Rac1 (Millipore, 1:2000), anti-anti-GTP-Rac1 (New East Bioscience, 1:1000), anti-GluR1 (Santa Cruz Biotech., 1:500), anti-TDP-43 (GeneTex, 1:4000) and anti-actin (Sigma, 1:10,000).

For quantitative RT-PCR analysis, the total RNA was isolated from the cultured hippocampal neurons using the Trizol method. The first strand cDNA synthesis was done using the Superscript RT (Invitrogen) and real time PCR was performed using the Light Cycler machine (Roche biochemical sciences) and appropriate primers corresponding to TDP-43, Rac1 and actin mRNAs.

Golgi staining of the brain sections

Golgi staining was carried out as described [4]. Briefly, 8 week-old wild type or Tg mice were anesthetized and perfused with PBS. The whole brain was removed, immersed in Golgi-Cox solution for 7 days, and then dehydrated in 30% glucose. Two hundred µm-thick sections were obtained by using a vibratome. The sections were collected serially, developed, fixed, cleared and coverslipped. For analysis of the spine density in the CA1 pyramidal neurons, the neurites were selected on the basis of the morphological criteria as described before [4].

Supplementary References

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4. Hayashi ML, Choi SY, Rao BS et al. (2004) Altered cortical synaptic morphology and impaired

memory consolidation in forebrain-specific dominant-negative PAK transgenic mice. Neuron 43: 773-787

5. Iguchi Y, Katsuno M, Niwa J et al. (2009) TDP-43 depletion induces neuronal cell damage through dysregulation of Rho family GTPases. J Biol Chem 284: 22059-22066
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8. Tsai KJ, Yang CH, Fang YH et al. (2010) Elevated expression of TDP-43 in the forebrain of mice is sufficient to cause neurological and pathological phenotypes mimicking FTLD-U. J Exp Med 207: 1661-1673