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## Low-level overexpression of wild type TDP-43 causes late-onset, progressive neurodegeneration and paralysis in mice [preprint]

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1 **Low-level overexpression of wild type TDP-43 causes late-onset, progressive**  
2 **neurodegeneration and paralysis in mice**

3  
4 Short title: **Low-level TDP-43 overexpression causes late-onset neurodegeneration in mice**

5  
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## Abstract

Modestly increased expression of transactive response DNA binding protein (*TDP-43*) gene have been reported in amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and other neuromuscular diseases. However, whether this modest elevation triggers neurodegeneration is not known. Although high levels of TDP-43 overexpression have been modeled in mice and shown to cause early death, models with low-level overexpression that mimic the human condition have not been established. In this study, transgenic mice overexpressing wild type TDP-43 at less than 60% above the endogenous CNS levels were constructed, and their phenotypes analyzed by a variety of techniques, including biochemical, molecular, histological, behavioral techniques and electromyography. The TDP-43 transgene was expressed in neurons, astrocytes, and oligodendrocytes in the cortex and predominantly in astrocytes and oligodendrocytes in the spinal cord. The mice developed a reproducible progressive weakness ending in paralysis in mid-life. Detailed analysis showed ~30% loss of large pyramidal neurons in the layer V motor cortex; in the spinal cord, severe demyelination was accompanied by oligodendrocyte injury, protein aggregation, astrogliosis and microgliosis, and elevation of neuroinflammation. Surprisingly, there was no loss of lower motor neurons in the lumbar spinal cord despite the complete paralysis of the hindlimbs. However, denervation was detected at the neuromuscular junction. These results demonstrate that low-level TDP-43 overexpression can cause diverse aspects of ALS, including late-onset and progressive motor dysfunction, neuroinflammation, and neurodegeneration. Our findings suggest that persistent modest elevations in TDP-43 expression can lead to ALS and other neurological disorders involving TDP-43 proteinopathy. Because of the predictable and progressive clinical paralytic phenotype, this transgenic mouse model will be useful in preclinical trial of therapeutics targeting neurological disorders associated with elevated levels of TDP-43.

72

## Introduction

73 ALS is a neurodegenerative disease that causes relentless, progressive loss of upper and lower  
74 motor neurons leading to paralysis and death. Approximately 90 percent of ALS are sporadic,  
75 whereas ~10 percent are familial. The mechanism of motor neuron degeneration in ALS is not  
76 yet fully understood. Mutations in many genes can cause or enhance the risk of ALS. These  
77 genes function in multiple cellular mechanisms, including protein homeostasis, RNA processing,  
78 inflammation regulation, cytoskeleton dynamics, and intracellular trafficking, thus suggesting  
79 that abnormalities in diverse pathways may lead to motor neuron neurodegeneration in ALS [1,  
80 2].

81 TDP-43 is an RNA binding protein whose intracellular inclusions are associated with >97% of  
82 ALS cases [3-6]. Present predominantly in the nucleus, TDP-43 regulates many RNA  
83 processes, including transcription, translation, splicing, and transport [7]. In addition, TDP-43  
84 regulates some miRNA processing, participates in stress granule formation [8], and suppresses  
85 cryptic exon expression [9]. TDP-43 function is essential for the survival of many types of cells  
86 in mammals; ubiquitous knockout of TDP-43 results in early embryonic death in mice [10-13].  
87 Mutations in the TDP-43 gene cause familial ALS [14-16]. These mutations increase the  
88 propensity of TDP-43 to misfold and aggregate [17] but have minimal effects on its mRNA  
89 splicing function or participation in stress granule formation [18-21]. Mutant TDP-43 proteins  
90 form insoluble intracellular aggregates in ALS patients [5]. Intriguingly, wild type TDP-43 protein  
91 also forms aggregates in sporadic ALS patients and in a large fraction of familial ALS that do not  
92 have TDP-43 mutations [3-5, 22]. This phenomenon of TDP-43 aggregation, designated as  
93 TDP-43 proteinopathy, is evident in motor neurons and oligodendrocytes [23, 24] and is thought  
94 to initiate motor neuron degeneration by both a loss of TDP-43 function and a gain of toxicity  
95 [22, 25, 26].

96 What drives wild type TDP-43 to proteinopathy is not understood. Several studies have  
97 suggested that modestly elevated levels of TDP-43 expression are associated with TDP-43  
98 proteinopathy in ALS and other diseases such as FTD and inclusion body myopathy, Paget's  
99 disease of the bone and frontotemporal dementia (IBMPFD) [27-30]. ALS-associated TDP-43  
100 mutations stabilize the TDP-43 protein or mRNA [19, 31-33]. TDP-43 levels are increased in  
101 cells with TDP-43 mutations from ALS patients and in mutant TDP-43 knock-in transgenic  
102 animals [15, 34, 35]. Remarkably, the mutant stability is inversely correlated with the disease  
103 onset age in ALS patients; the more stable the mutant, the earlier the disease onset [19]. These  
104 studies raise the question of whether a modestly elevated level of TDP-43 suffices to trigger  
105 progressive motor neuron degeneration and ALS.

106 To answer this question, a mammalian model with modestly elevated levels of TDP-43 should  
107 be informative, as such a model will closely mimic the modestly elevated levels in human ALS  
108 and create an opportunity to observe chronic TDP-43 toxicity. Previous mammalian models with  
109 TDP-43 overexpression have shown a wide range of phenotypes, including both motor and non-  
110 motor symptoms [36]. In general, the high TDP-43-expressing lines die rapidly after birth. Some  
111 of these mice showed TDP-43 intracellular inclusions and mild motor neuron loss [36]. The low  
112 TDP-43 expression lines develop subtle or no motor phenotypes with only mild cellular and  
113 molecular changes during the animal's lifespan [35-42]. These models notwithstanding, whether  
114 a modestly elevated level of TDP-43 within the range of the increases observed in human ALS  
115 can trigger neurodegeneration and ALS phenotypes remain uncertain.

116 In this study, we have constructed two TDP-43 transgenic lines with modest levels of TDP-43  
117 overexpression. Both lines developed motor weakness after ~500 days. The weakness  
118 eventually progressed to paralysis in a few months. Conversion of one line into homozygotes  
119 accelerated the disease. The homozygous mice developed paralysis during a period of 300 to  
120 430 days. Detailed analysis showed ~30% motor neuron loss in layer V cortex, oligodendrocyte

121 degeneration, demyelination, and neuroinflammation, but no motor neuron loss, in the spinal  
122 cord. However, denervation at the neuromuscular junction was detected, indicating the  
123 presence of distal motor axon degeneration. Thus, this mouse model has replicated several key  
124 features of ALS, including progressive motor dysfunction, paralysis, loss of upper motor  
125 neurons, oligodendrocyte degeneration, loss of myelin and neuroinflammation. These results  
126 demonstrate that a modest elevation of TDP-43 can trigger late-onset neurodegeneration and  
127 motor dysfunction and thus may play a causative role in human ALS and other neuromuscular  
128 conditions involving TDP-43 proteinopathy.

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## Materials and Methods

### 131 ***Transgenic mice***

132 A cDNA encoding mouse wild-type TDP-43 and EGFP linked by the internal ribosome entry site  
133 (*IRES*) was generated by standard PCR using the full-length mouse TDP-43 cDNA and EGFP  
134 as templates. The resulting cDNA (mTDP43-IRES-GFP) was cloned into the XhoI site of the  
135 MoPrp.Xho plasmid (ATCC#JHU-2) [43]. After the sequence verification and tests in cultured  
136 cells, the prp-mTDP43-IRES-EGFP construct was linearized by Not1 and injected into the  
137 pronuclei derived from FVB/NJ mice. The founder mice were screened by PCR using the  
138 primers complementary to the GFP and vector DNA sequence: forward  
139 TGCTGCTGCCCCGACAACCA and reverse ATAACCCCTCCCCAGCCTAGA. The positive  
140 founders were bred with wild type FVB/NJ mice, and the offspring were sacrificed and  
141 characterized for their expression of GFP in the CNS. Their brain and spinal cord were  
142 examined under a fluorescence microscope. The lines were terminated if 3 to 5 animals from  
143 the line showed no detectable GFP fluorescence. The lines surviving this screen were further  
144 analyzed for their GFP expression levels by immunoblot. Two lines, 19 and 42, were selected  
145 for a further screen for motor phenotypes. Both lines developed incompletely penetrant, late-  
146 onset motor dysfunction and paralysis. Line 19 was successfully converted to a homozygous  
147 line and further analyzed in detail. This line has been deposited at The Jackson Laboratory and  
148 will be available as Stock No. 031609.

### 149 ***Behavioral analysis***

150 Mice were housed in the University of Massachusetts Medical School animal facility managed  
151 by the Department of Animal Medicine. This facility is a specific pathogen-free (SPF) facility.  
152 Each cage housed 1-5 animals *ad libitum*. The rooms were maintained at 20-22°C and with a  
153 12-12 light-dark cycle. All the behavioral experiments were approved by IACUC and conducted  
154 according to University of Massachusetts Medical School policies and procedures regulating the

155 use of animals in research and the provisions of the PHS/NIH Guide for the Care and Use of  
156 Laboratory Animals.

157 **Home cage observation.** Mice were observed daily on weekdays for their general health and  
158 motor behavior, and their body weights monitored biweekly. The disease stages were assigned  
159 as follows: pre-symptomatic (pre-sym), slightly weak (swk), weak (wk), and paralysis (par). At  
160 the pre-sym stage (usually <10 months), the motor behavior was indistinguishable from the nTg  
161 mice. At the swk stage (at ~10 months), the mice showed slight foot-dragging in their gait. At the  
162 wk stage (~11-14 months), the foot-dragging became readily observable, and the movement  
163 became noticeably slowed. At the paralysis stage, two or more limbs became paralyzed, and  
164 the mouse was incapable of locomotion. The paralysis stage was the endpoint of the  
165 experiment, and the mouse was sacrificed for tissue harvesting.

166 **HomeCageScan.** To monitor the mouse behavior in their home cage continuously, we used the  
167 HomeCageScan system as previously described [44, 45]. Briefly, mice were housed individually  
168 in polycarbonate cages with minimal bedding (about 200 ml). A digital video camera was  
169 mounted on one side of the wall. Each mouse was recorded for 24h per week, with 12h daylight  
170 and 12h dim red light, and then returned to its cage with its littermates. Video data were  
171 analyzed by HomeCageScan software (Clever Systems, Reston, VA, USA) to quantify travel.  
172 Travel measures the overall motor/muscle functions by recording the distance traveled in  
173 meters.

174 **Accelerating rotarod.** Transgenic animals and age-matched controls were tested for time on  
175 accelerating rotarod from 12 to 72 RPM over three trials with a maximum time of 300s per trial  
176 at different age points. The longest time of three trials on the rotarod was recorded in seconds  
177 once the mice fell from the bar.

178 **Grip strength.** Transgenic animals and age-matched controls were tested for loss of four-limb  
179 grip strength using a grip strength meter at different time points. Mice were allowed to grip on a



180 horizontal metal wire grid with four limbs. They were gently pulled back by their tails with steady  
181 force until they release their grip from the grid. Peak tension was recorded from five consecutive  
182 trials.

### 183 ***Immunoblotting***

184 Mice under deep anesthesia were decapitated. The spinal cord, brain, and other tissues were  
185 quickly harvested, snap-frozen in liquid nitrogen, and stored at -80°C. For protein preparation,  
186 frozen tissues were homogenized in a homogenization solution containing 25 mM phosphate pH  
187 7.2, 1 mM EGTA, 1% SDS, 0.5% Triton X-100 and protease inhibitor mixture (Thermo Scientific)  
188 and heated at 95°C for 5 min. After clearing by centrifugation, protein concentration was  
189 measured using BCA assay (Pierce, Rockford, IL). The samples were heated in Laemmli buffer,  
190 and equal amounts of protein were loaded and resolved by SDS-PAGE. After transfer to  
191 nitrocellulose membranes, blots were blocked with 5% nonfat dry milk in PBST (0.25% Triton X-  
192 100 in PBS, pH 7.4) for 1 h, and then incubated with primary antibodies overnight at 4°C and  
193 then again with horseradish peroxidase–linked secondary antibodies (GE Healthcare) in PBST  
194 with 5% dry milk for 1 hour at RT. The dilutions and source of primary antibodies were as  
195 follows: GFP (Invitrogen G10362, 1:1000), rabbit polyclonal antibody raised to amino acids 394-  
196 414 of human TDP-43 (c-TDP43, custom made, 1:5000),  $\alpha$ -Tubulin (Sigma, T5168, 1:10000),  
197 CNPase (Cell Signaling, #5664s, 1:1000), MBP (Abcam, ab62631, 1:1000), MCT1 (Abcam,  
198 ab90582, 1:1000), NFkB-p65 (Cell signaling, #8242, 1:1000), and phosphorylated NFkB-p65  
199 (Cell signaling, #3033, 1:1000). Membranes were washed three times, and proteins were  
200 visualized after ECL (Pierce) treatment and detected by the LAS-3000 imaging system  
201 (Fujifilm).

### 202 ***Sedimentation assay***

203 Mouse lumbar spinal cords were homogenized using a handheld polytron for 20 sec in lysis  
204 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholic acid, 1% Triton X-100, 20 mM NaF, 1

205 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA) with protease inhibitor (1:100 dilution, P8340, Sigma, St Louis, MO,  
206 USA) and phosphatase inhibitor cocktails (Thermo Fisher). The homogenates (100 µL/sample)  
207 were centrifuged at 12,000g at 4°C for 5 min. The supernatants were moved to new tubes and  
208 measured for protein concentration as described above. The pellets were rinsed 3 times with  
209 the lysis buffer and resuspended in 20 µL 1X Laemmli buffer. Ten micrograms of protein from  
210 the supernatant were mixed with 2X Laemmli buffer. The supernatant sample and an equivalent  
211 volume of pellet sample were heated at 95°C for 5 min, cleared by centrifugation, and then  
212 resolved by SDS-PAGE. The gel was then immunoblotted, as described above.

### 213 ***RT-PCR and qRT-PCR***

214 For total RNA extraction, frozen tissues or sorted cells were homogenized in cold TRIzol  
215 reagent (Invitrogen) following the manufacturer's protocol. RNA was then reverse transcribed to  
216 cDNA using qScript cDNA SuperMix (Quanta BioSciences). For testing candidate splicing  
217 targets, RT-PCR amplification using between 33 and 37 cycles were performed from at least  
218 three nTg mice and three Tg mice. Products were separated on 2% agarose gels and visualized  
219 by staining with ethidium bromide and photographed. For qRT-PCR measurements of candidate  
220 gene targets, real-time PCR was performed on the cDNA using the primers for the targets. The  
221 PCR cycles were carried out in a Bio-Rad Real-Time PCR system (C1000 Thermal Cycler,  
222 Biorad), and the PCR product was detected using Sybr Green. The levels of target genes were  
223 standardized to the housekeeping gene GAPDH in individual animals and then further  
224 normalized to the mean  $\Delta$ CT of the wild type mice.

### 225 ***Immunofluorescence and immunohistochemistry***

226 Mice under deep anesthesia were transcardially perfused with cold PBS, followed by 4%  
227 paraformaldehyde in PBS. The perfused mice were then immersed in the same fixative at 4°C  
228 for another 24-48h. After fixation, tissues were immersed in PBS containing 30% sucrose at 4°C  
229 for 2–3 days. Tissues were then frozen in OCT freezing media (Sakura, Torrance, CA) and

230 stored at -20°C. Frozen sections were cut at 20 µm using a cryostat. For immunostaining,  
231 sections were incubated in the blocking solution (5% normal serum in PBS, pH 7.4) for 1 hour at  
232 room temperature (RT) and then incubated with a primary antibody in the blocking solution  
233 overnight at 4°C. The dilutions and source of primary antibodies were as follows: NeuN  
234 (Millipore MAB377, 1:200), calbindin (Millipore AB1778, 1:500), GFAP (Abcam Ab7260,  
235 1:1000), IBA1 (BioCare Medical CP290AB, 1:200), APC (EMD Bioscience OP80-100UG,  
236 1:200), GFP (Invitrogen G10362, 1:333), ChAT (Millipore AB1044P, 1:200), rabbit polyclonal  
237 antibody raised to amino acids 394-414 of human TDP-43 (custom made), TDP-43 (Encor  
238 biotechnology MCA-3H8, 1:250), NF-L (Cell Signaling, #2837, 1:100), CNPase (Cell Signaling,  
239 #5664s, 1:100), MBP (Abcam, ab62631, 1:100), NFκB-p65 (Cell Signaling, #8242, 1:100),  
240 activated caspase3 (R&D system, AF835, 1:1000). Sections were then washed 3 times for 5  
241 minutes each and incubated in the appropriate secondary antibody at room temperature for 90  
242 minutes. For immunofluorescence, the sections were washed 3 times in PBS for 5 minutes each  
243 and mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole  
244 (DAPI, Vector Laboratories) and sealed with nail polish. Images of the brain and spinal cord  
245 sections were taken with a confocal microscope (Leica).

246 For quantification of TDP-43 signal intensity in the nucleus and cytoplasm, sections were  
247 double-stained for TDP-43 and cellular markers. After staining, the cells were visualized and  
248 photographed using confocal microscopy. The cells in the ventral horn of the spinal cord were  
249 measured for their fluorescence intensity using the Nikon NIS Elements software. For each cell,  
250 the average fluorescence intensity was calculated. Cells on at least 5 different sections from  
251 each of the three or more mice per genotype were measured.

252 For immunohistochemistry, sections were washed 3 times in PBS containing 0.25% Tween 20  
253 and then stained following the manufacturer's instructions for Vectastain ABC kit, Elite PK-6100  
254 standard ImmPact™ DAB peroxidase Substrate kit SK-4105 (Vector Lab). The sections were

255 then mounted on slides and dried overnight at 55°C. After soaking in Xylenen 2 times for 2  
256 minutes each, the slides were sealed with Permount (Vector Lab).

### 257 ***Detection of demyelination***

258 To detection of demyelination, mice were fixed by transcardial perfusion using 4%  
259 paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium phosphate (pH 7.6). Tissues were  
260 further fixed by soaking in the same fixative at 4°C for 24 hours. Luxol fast blue staining was  
261 performed on 10-µm spinal cord or brain paraffin-embedded sections for demyelination.

### 262 ***Visualization and quantification of neurodegeneration***

263 Visualization and quantification of the cortical neurons were carried out as described previously  
264 {Aliaga, 2013 #1266}. Briefly, whole mouse brains were placed in 30% sucrose solution for 2  
265 days, frozen, and sectioned sagittally at 50 µm thickness. Layer V pyramidal neurons were  
266 counted using every ninth section, with a total of nine sections per half brain. Sections were  
267 mounted onto gelatin coated slides and stained with CTIP2 or Cresyl violet. Stereological  
268 counting was performed using Stereo Investigator software (MBF Bioscience, Williston VT).  
269 Counting was performed within the motor area of cortical layer V. Only pyramidal neurons with a  
270 soma greater than 15 µm in diameter were included. A single experimenter who was blinded to  
271 the genotype performed all counts.

272 For visualization of ventral root axons, mice were fixed by transcardial perfusion using 4%  
273 paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate (pH 7.6). Tissues were further  
274 fixed by soaking in the same fixative at 4°C for 24 hours. L4 and L5 roots attached to dorsal root  
275 ganglia were dissected and postfixated with 2% osmium tetroxide in 0.1 M phosphate (pH 7.6),  
276 dehydrated in a graded ethanol series, and embedded in Epon-Araldite resin. One-micron  
277 sections were stained with toluidine blue and examined and photographed by light microscopy.

278 For quantification of ventral horn motor neurons, lumbar enlargement of the fixed spinal cords  
279 was sectioned on a cryostat at 20 $\mu$ m thickness. Every other section was collected until a total of  
280 ten sections were collected from each spinal cord. The sections were stained with goat ChAT  
281 antibody at 4°C overnight. A secondary donkey anti-goat biotinylated antibody and a Vectastain  
282 ABC and DAB peroxidase Substrate kit (Vector Lab) were used to reveal motor neurons.  
283 Images of the spinal cord sections were taken using a Nikon microscope, and motor neuron  
284 numbers in the ventral horn region were counted manually from each section.

285 For muscle histology, isopentane in a container was pre-chilled with liquid nitrogen until the  
286 isopentane started to solidify at the bottom of the container. A fresh specimen isolated from  
287 gastrocnemius muscle was placed on a cork disc with a drop of OCT, which kept the muscle in  
288 the desired orientation. The specimen was frozen by immersion into the isopentane for about 5  
289 seconds and then stored at -20°C. The frozen tissue was sectioned using a cryostat and stained  
290 with Hematoxylin & Eosin (H&E).

### 291 ***Electromyography***

292 Mice were anesthetized by inhalation of isoflurane. Animals were placed immediately on a  
293 heating pad to maintain their core temperature at 37°C. Measurements were performed using a  
294 Cardinal Synergy electromyography (EMG) machine. A ground self-adhesive gelled surface  
295 electrode was placed over the tail. Potentials were recorded from several sites of the muscles of  
296 all four limbs with a concentric needle electrode (30G) using a gain of 50  $\mu$ V/division and a  
297 bandpass filter with low and high cut-off frequency settings of 20 and 10,000 Hz, respectively.  
298 The entire recording process took 15-20 minutes per mouse, after which the mice were  
299 euthanized by isoflurane overdose or used for tissue collection.

### 300 ***Visualization and quantification of neuromuscular junctions***

301 Animals are euthanized via an overdose of isoflurane, then transcardially perfused with PBS for  
302 2 minutes, followed by 4% paraformaldehyde for 5 minutes. Gastrocnemius muscles were  
303 dissected out and placed in 1.5% paraformaldehyde for 24 hours at 4°C. The muscles were  
304 then washed with PBS for 30min at 4°C and placed in 25% sucrose overnight at 4°C. Muscles  
305 were embedded in OCT medium, frozen rapidly, and stored at -80°C. Sections were cut at  
306 35nm thickness using a Leica Cryostat, placed on Superfrost Plus slides, and stored at -80°C.  
307 Slides were allowed to defrost for 30 minutes before use. Slides were washed 3 times with PBS  
308 for 5 minutes and then 3 times with 4% Triton X-100 for 5 minutes. Sections were blocked using  
309 10% donkey serum in PBS for 3.5 hours at room temperature. A primary antibody solution of  
310 Rabbit anti-synaptophysin (ThermoFisher, 1:1000) and rabbit anti-Neuronal class III Beta-  
311 Tubulin (Biolegend, 1:1000) diluted in blocking solution was applied for 24 hours at 4°C. Slides  
312 were washed again with PBS. A secondary antibody solution of Alexa-488nm-labelled Donkey  
313 anti-Rabbit (ThermoFisher, 1:500) and Alexa-555nm  $\alpha$ -Bungarotoxin (ThermoFisher, 1:500)  
314 diluted in PBS was applied overnight at 4°C in the dark. Slides were imaged on a Nikon  
315 microscope. Neuromuscular junctions were then counted and quantified as innervated or  
316 denervated Based on nerve occupancy of the endplates. Those with >50% occupancy was  
317 counted as innervated and those with <50% were counted as denervated.

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## Results

### 321 ***Prion promoter drives TDP-43 expression primarily in the CNS and causes age-*** 322 ***dependent, progressive weakness, and paralysis***

323 To determine the effects of an elevated level of TDP-43, we constructed a transgene in which  
324 the mouse prion promoter (Prp) [43] drives the expression of wild type murine TDP-43 cDNA.  
325 We employed the authentic TDP-43 sequence to avoid any possible effects of a tag. GFP was  
326 co-expressed from an internal ribosomal entry site (IRES) to monitor the transgene expression  
327 (S1A Fig). The founder lines were screened for their expression of GFP in the CNS. Two lines  
328 (lines 19 and 42) were obtained. Both expressed similar levels of GFP and TDP-43 (S1B Fig).  
329 Western blots of tissues from various organs showed that the transgene was predominantly  
330 expressed in the CNS (S1C, D Fig). Despite the readily detectable GFP expression, changes in  
331 TDP-43 levels were hardly noticeable in these lines compared with non-transgenic (nTg) mice  
332 (S1B Fig), possibly as a result of TDP-43 autoregulation, as previously reported [46]. A small  
333 cohort from each of the two lines was monitored for up to 750 days. Five of the ten mice from  
334 line 19 and four of the five mice from line 42 developed motor deficits ending in full paralysis  
335 (S1E, F Fig). The symptomatic mice also showed progressive weight loss towards the paralysis  
336 stage (S1G Fig), similar to other animal models for ALS [36].

337 To facilitate the analysis of these mice, we sought to elevate the gene dosage by making  
338 homozygous lines. We were unable to generate homozygous mice from line 42 but succeeded  
339 from line 19. As anticipated, the homozygous mice developed motor symptoms earlier than the  
340 original hemizygous line and became paralyzed before 400 days (Fig. 1A, B; S1 Video). This  
341 phenotype was completely penetrant. Males developed paralysis at younger ages ( $\sim 356 \pm 19$   
342 days) than the females ( $\sim 385 \pm 26$  days) (Fig. 1B). The motor symptoms progressed over  
343 approximately three to five months before ending in paralysis based on several different  
344 behavioral measures, including body weight, travel velocity, grip strength, and rotarod

345 performance (Fig. 1C-F, S1 Video). Given the accelerated and completely penetrant phenotype,  
346 we focused on these mice for further analysis and referred to the TDP-43 line 19 homozygous  
347 transgenic mice as TDP-43 transgenic (Tg) mice in the following text.

348 **Fig. 1. The TDP-43 homozygous transgenic (Tg) mice developed progressive motor**  
349 **phenotypes.** (A) An example of Tg mice with hind limb paralysis (left). The mouse on the right  
350 was a non-transgenic (nTg) control. (B) Kaplan-Meier plot of Tg mice (37 males and 30 females)  
351 that are not paralyzed. (C) The bodyweight of the Tg mice peaked at ~9 months of age and then  
352 declined until the paralysis stage. The values were averaged from 10 to 21 animals per group.  
353 (D) The home cage average travel velocity within a 24-hour period at various ages. The Tg mice  
354 went through a hyperactive stage before developing weakness and paralysis. (E) The Tg mice  
355 developed progressively weaker 4-limb grip strength after 250 days of age. (F) The Tg mice  
356 showed a declining rotarod performance after 200 days of age. Each data point in D, E, and F  
357 was an average of 16 animals.

358 As expected, the increased gene dosage in the homozygous mice increased the expression of  
359 the transgene compared with the hemizygous mice, as shown by the Western blots for GFP and  
360 TDP-43 (Fig. 2A). The hemizygous mice expressed TDP-43 at ~10% above the nTg level in the  
361 spinal cord and ~20% above in the frontal cortex (Fig. 2B). By comparison, the homozygous  
362 mice expressed ~30% above the nTg level in the spinal cord and ~40% above in the frontal  
363 cortex (Fig. 2B). Higher levels of mRNA were observed. The hemizygous mice expressed TDP-  
364 43 at ~3 and ~4 fold of the nTg levels in the spinal cord and the frontal cortex, respectively. By  
365 comparison, the homozygous mice expressed ~5 and ~8 fold of the nTg levels in the spinal cord  
366 and the frontal cortex, respectively (Fig. 2C).

367 **Fig. 2. Transgene expression in the CNS of the hemizygous (Hemi.) and homozygous**  
368 **(Homo.) Tg mice.** (A) A modest increase of TDP-43 protein in the frontal cortex (FC),  
369 cerebellum (CB), brainstem (BS), cervical (CSC), and lumbar spinal cords (LSC). Each lane



370 represents one mouse. Notice that the 35-KD proteolytic fragment of TDP-43 was only detected  
371 in the transgenic mice but not in nTg controls. (B) Quantification of TDP-43 protein in (A) in FC  
372 and LSC. (C) Quantification of TDP-43 mRNA in FC and LSC. The number of animals used for  
373 FC quantification: nTg, 8; Hemi, 9; Homo, 11. For LSC: nTg, 9; Hemi, 12; Homo, 16. Error bars  
374 are standard deviation. (D) Comparing the alternative splicing patterns in the Tg mice vs. TDP-  
375 43 knockdown mice [45]. The PCR primers and the exon numbers are listed in S1 Table. (E) A  
376 sagittal section from a Tg mouse brain and a cross-section from either Tg or nTg mouse spinal  
377 cord were stained for GFP. Notice that the broad GFP staining throughout the Tg brain and  
378 spinal cord but not in the nTg. (F) Western blot showing stable TDP-43 protein levels in the  
379 TDP-43 Tg mice throughout different ages. The arrow point to the 35-KD fragment that is only  
380 present in the Tg mice.

381 TDP-43 is a gene-splicing modulator. To determine whether the modest elevation of TDP-43  
382 level in the Tg mice impacted TDP-43 function, we measured the splicing patterns of some  
383 TDP-43-regulated mRNAs [47, 48]. For comparison, we also examined splicing patterns of the  
384 same target mRNAs in the TDP-43 knockdown (KD) mice [45]. Of the fifteen alternatively  
385 spliced genes that we measured, seven changed in the opposite direction in the TDP-43 Tg  
386 mice compared to the TDP-43 KD mice, with exon exclusion increased the Tg mice but  
387 decreased in the KD mice (Fig. 2D, red genes); four genes changed in the same direction, with  
388 exon exclusion enhanced in both the Tg and KD mice (Fig. 2D, blue genes); the final four genes  
389 did not change significantly in the Tg mice, but showed increased exon exclusion in the KD mice  
390 (Fig. 2D, black genes). Overall, the splicing modulation in our Tg mice was biased toward exon  
391 exclusion, consistent with the gain of TDP-43 function as reported in the literature [39, 47].

392 To determine where the transgene is expressed in the CNS, we stained brain and spinal cord  
393 sections for GFP. We observed a broad expression pattern in all regions of the CNS (Fig. 2E).  
394 The elevated level of expression was also sustained throughout the lifespan of the mice (Fig.

395 2F). To determine the cell types that express the transgene, we conducted double  
396 immunofluorescence staining. In spinal cord, we observed high levels of GFP in  
397 oligodendrocytes and astrocytes, and low levels in microglia and neurons, including motor  
398 neurons (Fig. 3). In brain, we observed relatively high levels of GFP in oligodendrocytes,  
399 astrocytes and neurons, and low levels in microglia (Fig. 3).

400 **Fig. 3. The Tg mice expressed transgenes in multiple different cell types in the spinal**  
401 **cord and the brain.** In the spinal cord, the expression was relatively strong in oligodendrocytes  
402 (ODC) and astrocytes but weak in microglia and neurons, including motor neurons. In the frontal  
403 cortex (top four rows under the brain label), the transgenes are expressed strongly in ODC,  
404 astrocytes, and neurons (arrows) but weakly in microglia. Sections were double immunostained  
405 for GFP and various cellular marks as indicated. The transgenes were weakly expressed in  
406 Purkinje cells compared with the surrounding neuropils in the cerebellum. All panels are in the  
407 same magnification. All animals used are pre-symptomatic and less than 100 days old.

408 The increased TDP-43 expression may perturb the nuclear and cytoplasmic distribution of TDP-  
409 43. Because such perturbation may impact neurodegeneration [49], we quantified the nuclear  
410 and cytoplasmic TDP-43 staining intensities. TDP-43 was increased in both the nucleus and  
411 cytoplasm in all cell types that we measured (Fig. 4B). The overall increase was larger in glial  
412 cells than in neurons. In the nucleus, TDP-43 was up by ~150-220% in glial cells, compared  
413 with up by ~30-50% in neurons (Fig. 4B). Likewise, in the cytoplasm, TDP-43 was increased by  
414 ~90-145% in glial cells, compared with an increase of ~80-90% in neurons. Interestingly, the  
415 cytoplasmic increase was larger than the nuclear increase in neurons but was smaller in glial  
416 cells (Fig. 4B), resulting in a decreased cytoplasmic-to-nuclear TDP-43 in glial cells but an  
417 increased ratio in neurons compared with nTg mice (Table 1). Despite the increase, we did not  
418 observe TDP-43 aggregates in any disease stages, even though sedimentation experiments

419 showed a ~25% increase in the detergent-insoluble TDP-43 and ubiquitinated proteins in the Tg  
 420 mice at the end disease stage (S2 Fig.), suggesting a modest increase in protein aggregation.

421 **Fig. 4. TDP-43 distribution in glial cells and neurons.** (A) Mouse spinal cords were immuno-  
 422 stained with cellular markers for oligodendrocytes (APC), astrocytes (GFAP), microglia (IBA1),  
 423 motor neurons (ChAT), and pan neurons (NeuN). For TDP-43 staining, images of both regular  
 424 (Reg.) and long-exposure (Long exp.) are shown. The long-exposure images were used to  
 425 visualize the cytoplasmic signal and quantify TDP-43 staining intensity in the cytoplasm. All  
 426 images are in the same magnification. (B) Quantification of staining intensity of TDP-43 in the  
 427 nucleus and cytoplasm of various cell types show in (A). Each symbol represents measurement  
 428 from one cell. Each column of symbols represents measurements from one mouse. The filled  
 429 symbols represent averages for each genotype. The changes of averages in percentage in the  
 430 Tg mice and the statistical p values are shown on the graphs. Student's t test is used to obtain  
 431 the p values. n = 3 for nTg and 5 for Tg mice, respectively.

**Table 1. Ratio of TDP-43 cytoplasmic levels to the nuclear levels**

	Oligodendrocyte	Astrocyte	Microglia	Motor N	Neuron
nTg (n = 3)	0.226±0.019	0.229±0.091	0.306±0.077	0.135±0.006	0.162±0.008
Tg (n = 5)	0.217±0.029	0.158±0.022	0.203±0.036	0.178±0.019	0.203±0.026
% Tg change from nTg	-3.8%	-31.1%	-33.7%	32.7%	25.6%
p (t test)	0.6298	0.2649	0.1326	0.0048	0.0195

Motor N = motor neuron

432

433 ***TDP-43 mice develop severe white matter degeneration and oligodendrocyte injury in the***  
 434 ***low spinal cord***

435 To determine the pathological basis of the clinical phenotype, we examined the spinal cord  
436 throughout the various disease progression stages in the Tg mice. The most conspicuous  
437 pathological feature was white matter degeneration. Luxol fast blue staining revealed normal  
438 myelination at age five months, but the staining became slightly pale at eight months. At ten  
439 months, the staining became paler (Fig. 5A). At the end-stage (12 months), the staining was  
440 completely lost in the ventral spinal cord (Fig. 5A, D and S3A Fig), indicating loss of myelin,  
441 which we confirmed by electron microscopy (S3B Fig). The myelin loss was accompanied by  
442 increasing astrogliosis (Fig. 5B, E) and microgliosis (Fig. 5C, F).

443 **Fig. 5. The Tg mice developed age-dependent myelin degeneration and gliosis in the**  
444 **spinal cord.** (A) Late-onset, progressive demyelination, (B) astrogliosis and (C) microgliosis in  
445 the lumbar spinal cord of the Tg mice. The right panels are magnified views of boxed areas in  
446 the left panels. (D, E, F) Enlarged views of ventral horn stained with Luxol Fast Blue and Cresyl  
447 violet, GFAP and Iba1, respectively. Shown are images representative of five or more animals  
448 for each genotype and at each time point.

449 Myelin loss could be associated with injury in oligodendrocytes as a result of TDP-43  
450 overexpression. Therefore, we doubly stained the spinal cord sections for oligodendrocyte  
451 marker APC and cell death marker activated caspase 3. We found an increasing association of  
452 activated caspase 3 and oligodendrocytes during the progression of the clinical phenotypes  
453 (Fig. 6A), and a reduction in oligodendrocyte-associated proteins, including CNPase, MBP, and  
454 MCT1 (Fig. 6B). Staining of CNPase in the white matter was also broadly lower than the nTg  
455 mice (Fig. 6C).

456 **Fig. 6. Oligodendrocyte pathology in TDP-43 mice.** (A) Age-dependent and progressively  
457 increased activated caspase 3 signals are associated with oligodendrocytes (arrows) in the  
458 spinal cord of Tg mice. (B) Reduced myelin protein levels in the Tg mice with paralysis. M =  
459 month, CNPase = 2',3'-cyclic-nucleotide 3'-phosphodiesterase, MBP = myelin basic protein,

460 MCT1 = monocarboxylate transporter 1. (C) A reduced staining intensity of CNPase in the  
461 paralyzed Tg mice compared with the nTg mice.

462 ***TDP-43 mice show no motor neuron loss but a mild muscle denervation***

463 The progressive clinical motor phenotype suggests the presence of motor neuron injury. By  
464 Nissl staining and choline acetyltransferase (ChAT) staining, we did not observe gross  
465 morphological deviations from the controls (Figs. 5D, 7A). Quantification of ChAT-positive  
466 neurons in the ventral horn also did not reveal a significant difference between the Tg mice and  
467 the nTg mice (Fig. 7B). To verify this result, we examined and quantified ventral root axon  
468 numbers. We did not observe a significant change in the number of ventral root axons in the Tg  
469 mice from the nTg mice (Fig. 7C-E). Although we could not detect any motor neuron number  
470 changes, the Tg mice might have been at an early stage of motor neuron degeneration, which  
471 had not yet been reflected in the motor neuron numbers. To examine this possibility, we stained  
472 for activated caspase 3. We did not detect an association between the activated caspase 3 with  
473 motor neurons, although caspase staining of surrounding cells, possibly glial cells, were  
474 increased (Fig. 7F).

475 **Fig. 7. No spinal motor neuron loss in the ventral horn of the lumbar spinal cord.** (A)  
476 ChAT staining of the ventral horn. (B) Quantification of ChAT-positive cells in the ventral horn of  
477 the spinal cord. ChAT-positive cells in the ventral horn were counted in 8-10 sections from the  
478 lumbar L4-5 levels of each animal. The average number per section was calculated from each  
479 animal. Four animals in each group were further averaged and shown here. Error bars  
480 represent standard deviation. (C) The axons in the ventral root and dorsal root remain normal.  
481 (D) High magnification of the boxed area in the dorsal and ventral root at the paralysis stage  
482 revealed no axon degeneration. (E) Quantification of dorsal and ventral roots showed no  
483 difference between Tg and nTg mice. (F) Immunofluorescence staining for activated caspase 3

484 show increased staining of the neuropils, but no motor neuron staining was detected. sym =  
485 symptomatic stage, par = paralyzed stage.

486 Despite the preservation of motor neuron cell bodies and their proximal axons, the distal axons  
487 and neuromuscular junction could be affected. To test this possibility, we examined muscle  
488 morphology and physiology. We did not observe muscle fiber atrophy (Fig. 8A, B). By needle  
489 electromyography (EMG), we observed that ~half (3 of 5 homozygotes and 1 of 3 hemizygotes)  
490 of the paralyzed mice had a completely normal EMG pattern that was indistinguishable from the  
491 control nTg mice (Fig. 8C1). The other half of the paralyzed mice showed various degrees of  
492 positive sharp waves (PSWs) in different muscles ranging from normal (Fig. 8C1) to single and  
493 to multiple PSWs (Fig. 8C2, 3). Quantification of endplate nerve occupancy in the  
494 gastrocnemius muscle unveiled an average of ~30% denervation in the paralyzed homozygous  
495 Tg animals compared with ~10% denervation in the age-comparable control nTg mice (Fig. 8D).

496 **Fig. 8. Denervation at the neuromuscular junction.** (A) Examples of cross-sections of  
497 gastrocnemius muscle from 12-month old nTg (left), 10-month old (middle), and 12-month old  
498 paralyzed TDP-43 Tg mice (right). (B) Quantification of muscle fiber cross-section sizes. Muscle  
499 sizes from individual animals were averaged. The average values from four animals were  
500 further averaged and depicted. Error bars are standard errors. The p values were obtained by  
501 comparing the nTg with the Tg mice using student t test. (C) Examples of electromyographic  
502 (EMG) traces from an nTg mouse (trace 1) and a paralyzed Tg mouse (trace 2 and 3). (D)  
503 Quantification of denervated muscle endplates. n = 4 in both Tg and nTg groups. Statistical  
504 comparison between the Tg and nTg groups was conducted using Wilcoxon Non-parametric  
505 method.

#### 506 ***TDP-43 mice show elevated neuroinflammation in their spinal cord***

507 Demyelination and gliosis are commonly associated with neuroinflammation [50]. Therefore, we  
508 examined the state of neuroinflammation in the TDP-43 transgenic mice. We found that the

509 mRNAs of neuroinflammation-associated genes such as TNF- $\alpha$  and NF- $\kappa$ B p65 subunit were  
510 increased along with the disease progression (Fig. 9A). Furthermore, the p65 protein and its  
511 phosphorylation were also increased in parallel with clinical symptom development in the Tg  
512 mice compared with the nTg mice (Fig. 9B). By immunostaining, the increased p65 was  
513 associated with oligodendrocytes (Fig. 9C, arrows). These changes suggested the presence of  
514 neuroinflammation in the TDP-43 transgenic mice.

515 **Fig. 9. Activation of NF- $\kappa$ B pathway in the Tg mice.** (A) TNF- $\alpha$  and p65 mRNA levels were  
516 dramatically elevated in the symptomatic Tg mice, and the peak was correlated with paralysis.  
517 The value of nTg mice is an average from eight animals with the age range from 102 days to  
518 436 days. The nTg mice did not show an age-dependent change and therefore grouped  
519 together. Student t tests with Bonferroni correction were used to compare between the Tg and  
520 the nTg mice at different ages. n = 4-8 at each age for both groups. \*\* =  $p < 0.01$ , \*\*\*\* =  $p <$   
521  $0.0001$ . (B) Western blot analysis showed both the phosphorylated p65 and the overall p65  
522 levels were increased near symptomatic onset and paralysis stage in the Tg mice. (C)  
523 Immunostaining for p65 and markers of glial cells show localization of the increased p65 in the  
524 oligodendrocytes (arrows) but not in astrocytes and microglia in the Tg mice.

525 To further confirm this observation, we examined additional molecular markers for  
526 neuroinflammation. The expression of NADPH oxidase (NOX), which is involved in inflammation  
527 and oxidative stress, was dramatically increased. The three mRNAs of its subunits, gp91<sup>phox</sup>,  
528 p22<sup>phox</sup>, and p67<sup>phox</sup>, were increased by ~600, ~80, and ~9 fold, respectively (Fig. 10A-C). Two  
529 genes involved in the production of prostaglandins, the inducible cyclooxygenase 2 (COX-2)  
530 and hemopoietic prostaglandin D synthase (HPDGS), were increased (Fig. 10D, E). GLT-1, an  
531 astrocytic glutamate transporter, was decreased at the end-stage (Fig. 10F). LCN2 and IL-6,  
532 two proinflammatory and neurotoxic cytokines, were also highly upregulated (Fig. 10G-I).  
533 Concomitant with these changes, the protein oxidation levels were increased (Fig. 10J).

534 Although not all the markers that we measured showed an increase (S4 Fig), the changes in the  
535 multiple neuroinflammation-associated markers suggest that the Tg mice undergo  
536 neuroinflammation in the spinal cord.

537 **Fig. 10. Age-dependent alterations in the expression of neuroinflammatory markers. (A-C)**

538 A dramatic increase in subunits of NADPH oxidase 2 (Nox2) gp91phox, p22, and p67 mRNA  
539 expression in late stages of motor dysfunction in the Tg mice. (E, F) Increased mRNA  
540 expression of prostaglandin-producing enzymes cyclooxygenase 2 (COX-2 or PTGS2) and  
541 hematopoietic prostaglandin D synthase (HPGDS), respectively. (G) Decreased expression of  
542 GLT-1 mRNA. (H, I) Increased expression of lipocalin 2 (LCN2) mRNA and protein,  
543 respectively. (J) Increased expression of IL-6 mRNA. (K) Western blot for dinitrophenyl (DNP)  
544 hydrazone derived from carbonylated proteins. Student t tests with Bonferroni correction were  
545 used to compare between the Tg and the nTg mice at different ages in (A-H) and (J). n = 3-8 at  
546 each age for both Tg and nTg groups. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

547 ***TDP-43 mice show a mild white matter degeneration in CNS areas beyond the lumbar***  
548 ***spinal cord***

549 Because the TDP-43 transgenic mice expressed an elevated level of TDP-43 in the forebrain  
550 (Figs. 2A, E; 3), it is possible that the upper motor neuron degeneration could contribute to the  
551 paralysis phenotype. To examine this possibility, we investigated whether there was  
552 neurodegeneration in the forebrain. The brain weight in the TDP-43 mice did not differ from  
553 those of the nTg mice (S5 Fig), suggesting no widespread neuronal loss in the brain. Staining  
554 for myelin at different CNS levels showed that demyelination was most severe in the lumbar  
555 spinal cord and became progressively less severe towards the rostral CNS (Fig. 11A). The  
556 myelin staining was almost absent in the lumbar spinal cord, and paler compared to the nTg  
557 mice in the cervical spinal cord and lower brainstem (Fig. 11A). At the level of pons and above,  
558 no apparent difference in the staining was noticeable between the nTg and Tg mice (Fig. 11A).



559 At the cervical spinal cord level, the myelin staining intensity throughout the different ages  
560 showed a late-onset, progressive demyelination (Fig. 11B), a pattern similar to the lumbar  
561 region (Fig. 5A) though less severe. These results indicate that the overt demyelination in the  
562 Tg mice is mostly confined to below the brainstem.

563 **Fig. 11. A survey of demyelination in the CNS.** (A) The severity of myelin degeneration  
564 showed a caudal to rostral gradient in the CNS, with the lumbar spinal cord (LSC) being the  
565 most severely affected, followed by the cervical spinal cord (CSC), lower brainstem, upper  
566 brainstem, and corpus callosum (cc). py = pyramidal track, cp = cerebellar peduncle, ic =  
567 internal capsule and ec = external capsule. (B) The progression of demyelination in the cervical  
568 spinal cord. The panels on the right are enlargements of boxed areas in the panels on the left.  
569 Notice the degree of demyelination is less severe than in the lumbar spinal cord at 12 months  
570 old (compare with Fig. 5A and also between LSC and CSC in A of this figure). Shown are  
571 images representative of five or more animals for each genotype and at each age point.  
572 We also surveyed gliosis in various regions of the brain in the paralyzed mice. We found  
573 elevated levels of astrogliosis and microgliosis in all regions, including the cerebral cortex,  
574 hippocampus, corpus callosum, internal capsule, cerebellum, and brainstem (Fig. 12),  
575 suggesting that elevated expression of TDP-43 provokes an inflammatory response by glial  
576 cells.

577 **Fig. 12. Mild but widespread gliosis in the upper brain regions in Tg mice.** Staining for  
578 GFAP and Iba1 in the brain showed mildly elevated levels of astrogliosis and microgliosis in  
579 various regions of the CNS in the Tg mice, including the cerebral cortex, hippocampus (HPC),  
580 corpus callosum (CC), internal capsule (IC), cerebellum (CB) and brainstem (BS).

581 ***TDP-43 mice develop neuronal loss in the motor cortex***

582 Because TDP-43 was expressed in both neurons and glia in the cortex (Fig. 3), we investigated  
583 whether there was a neuronal loss in the motor cortex. Ctip2 staining revealed dramatically  
584 lowered staining intensity compared with the nTg control (S6A Fig). A quantification of Ctip2-  
585 positive neurons in layer V showed a near 50% loss (S6B Fig). However, because there was a  
586 significant loss of Ctip2 expression in the motor cortex of the paralyzed mice (S6C Fig), the loss  
587 of Ctip2 expression might have caused the lowered neuronal counts. Therefore, we applied  
588 Nissl staining (Fig. 13A, B) and repeated the quantification of neurons in layer V cortex. This  
589 approach revealed ~30% neuronal loss (Fig. 13C). These results indicate that there is motor  
590 neuron loss in layer V of the motor cortex.

591 **Fig. 13. Visualization and quantification of Nissl-stained neurons in the motor cortex.** (A)  
592 Nissl stained motor cortex in nTg and paralyzed Tg mice. (B) Enlarged views of layer V motor  
593 cortex. (C) quantification of large (>15  $\mu\text{m}$  in diameter) Nissl-stained neurons in layer V. P value  
594 was derived from Student's t test. n = 4 for nTg and 3 for Tg mice.

595

596

## Discussion

597 This study has established a line of TDP-43 transgenic mice that express exogenous TDP-43  
598 protein at modestly elevated levels. Respectively for hemizygous and homozygous mice, the  
599 levels are ~10% and ~30% above the nTg level in the spinal cord and ~20 and ~40% increase  
600 frontal cortex (Fig. 2). These levels are comparable with the increase reported in human ALS  
601 and FTD [15, 28, 30, 34]. These TDP-43 mice show several key features of ALS, including late-  
602 onset progressive motor dysfunction ending in paralysis in mid-life, oligodendrocyte injury,  
603 demyelination, neuroinflammation, distal motor axon degeneration in the periphery, and cortical  
604 motor neuron loss. Thus, this transgenic mouse model demonstrates the multiple long-term  
605 adverse consequences of low-level TDP-43 overexpression *in vivo* and demonstrates that such  
606 low-level elevation of TDP-43, as observed in ALS and other neurodegenerative conditions, can  
607 trigger neurodegenerative clinical phenotypes.

608 How low-level overexpression of TDP-43 causes the late-onset neurotoxicity remains unknown.  
609 Pathological studies have consistently detected various forms of TDP-43 aggregates in ALS and  
610 FTD CNS tissues [51], suggesting an association between TDP-43 aggregation and the  
611 pathogenesis of these diseases. In vitro studies have shown that overexpression of TDP-43 can  
612 induce cytoplasmic liquid-liquid phase separation (LLPS), which, upon stress, transform into  
613 permanent aggregates. These aggregates can then draw TDP-43 out of the nucleus and kill the  
614 cell [52, 53]. However, in vivo models have shown divergent results. Some models show  
615 cytoplasmic TDP-43 aggregates either with or without nuclear TDP-43 depletion [37, 54, 55].  
616 Other studies suggest that neither TDP-43 aggregation nor its nuclear depletion is required for  
617 neurodegeneration [39, 56]. Our results present a nuanced picture. Although we did not observe  
618 cytoplasmic aggregates and nuclear depletion in cells, we detected an increase in the  
619 detergent-insoluble TDP-43 and high molecular-weight ubiquitinated protein species (S2 Fig.),  
620 suggesting there is TDP-43 protein aggregation, albeit modest, in the TDP-43 mice. These

621 results suggest that a modest elevation of TDP-43 levels can lead to TDP-43 protein  
622 aggregation, which could contribute to neurodegeneration in the TDP-43 mice.

623 Previous studies suggest that TDP-43 aggregation and nuclear depletion may lead to gain of  
624 toxicity as well as loss of function that ultimately cause cellular degeneration, including both  
625 neurons and oligodendrocytes [22, 23, 26, 57-61]. Analysis of mRNA splicing in mouse models  
626 has shown that a loss of TDP-43 function increases cryptic exon inclusion, whereas a gain of  
627 TDP-43 function leads to an increase in skiptic exons [9, 39]. Both the increases in the cryptic  
628 exon inclusion and the skiptic exons have been observed in human ALS samples, supporting  
629 the dual gain- and loss-of-function hypothesis [9, 39]. Consistent this hypothesis, both loss- and  
630 gain-of-function mouse models, including the model described in this report, produce motor  
631 neuron degeneration and ALS-like phenotypes [36, 39, 45, 62, 63], suggesting that each type of  
632 these models represent one aspect of the mechanism.

633 An intriguing observation in this study is that different cell types may be responding to TDP-43  
634 overexpression differently. In oligodendrocytes, TDP-43 overexpression elicited similar  
635 percentage of increases in nucleus and cytoplasm (Fig. 4B), thereby maintaining the same  
636 cytoplasmic-to-nuclear TDP-43 ratio as in the nTg mice (Table 1). In astrocytes and microglia,  
637 the percentages of nuclear increase were approximately twice the cytoplasmic increases (Fig.  
638 4B). As a result, the cytoplasmic-to-nuclear TDP-43 ratios were reduced, though not statistically  
639 significant (Table 1). By contrast, in motor neurons and non-motor neurons, the percentage of  
640 cytoplasmic increases was approximate twice the nuclear increases (Fig. 4B). Consequently,  
641 the ratio of cytoplasmic-to-nuclear TDP-43 was increased (Table 1). These observations  
642 suggest that neurons, particularly motor neurons, may handle the TDP-43 overexpression  
643 differently. It will be interesting to determine whether this observation can be replicated in other  
644 TDP-43 overexpression models in future experiments because of the potentially detrimental  
645 effects of an increased TDP-43 level in the cytoplasm [49].

646 Non-cell autonomous mutant toxicity is a well-established phenomenon in motor neuron  
647 degeneration provoked by mutant SOD1 [64]. Expression of mutant SOD1 in glial cells,  
648 including astrocytes, microglia and oligodendrocytes, accelerates motor neuron degeneration in  
649 vivo [65-69]. Glial cells expressing mutant SOD1 can also promote motor neuron degeneration  
650 in co-cultures in vitro [70-72]. Furthermore, mutant SOD1-expressing astrocytes and microglia  
651 secrete neuroinflammatory factors that are toxic and capable of killing motor neurons [66, 70,  
652 71]. Mutant SOD1 expression in oligodendrocytes leads to cellular dysfunction, rendering them  
653 incapable of supporting neuronal axons [68, 69]. The evidence strongly supports the view that  
654 the glial expression of mutant SOD1 significantly contributes to motor neuron degeneration.

655 However, whether this is the case for TDP-43 is less clear. Astrocyte-specific expression of  
656 mutant TDP-43 in rats induces motor neuron degeneration as the mutant expression in motor  
657 neurons [73, 74]. However, mutant TDP-43-expressing astrocytes fail to show toxicity in motor  
658 neuron-astrocyte coculture or after being transplanted in rat spinal cord [75, 76]. Our TDP-43  
659 transgenic mice showed two neurodegeneration patterns, one in the spinal cord and the other in  
660 the frontal cortex. In the spinal cord, the TDP-43 transgene was expressed highly in glial cells  
661 but lowly in neurons (Figs. 3, 4). The primary pathology was oligodendrocyte injury,  
662 demyelination, gliosis, and neuroinflammation (Figs. 5, 6, 9-11, and S3 Fig). However, no motor  
663 neuron loss was detected (Fig. 7). In the motor cortex, the TDP-43 transgene was expressed in  
664 both neurons and glial cells (Fig. 3). Approximately 30% of large pyramidal neurons were lost in  
665 layer V (Fig. 13 and S6 Fig). These results suggest that motor neurons can tolerate the  
666 detrimental effects invoked by modestly elevated levels of TDP-43 in their neighboring cells so  
667 long as the TDP-43 levels in themselves are maintained at relatively low levels. Even so, the  
668 tolerance of motor neurons to the injuries to their neighboring cells and neuroinflammation is  
669 probably limited, as illustrated by the presence of distal motor axon degeneration and  
670 neuromuscular denervation (Fig. 8). Because we have to sacrifice the mice at their paralysis

671 stage, we do not know whether letting the disease progress further will eventually lead to the  
672 loss of the lower motor neurons. In any case, our results are consistent with contributions to  
673 motor neuron toxicity from both cell-autonomous and non-cell-autonomous sources.

674 A unique feature of our TDP-43 mice, compared with other established TDP-43 mouse lines, is  
675 the development of a fully penetrant progressive disease course (Fig. 1, S1 Video). The  
676 phenotype of late-onset, slowly progressing motor dysfunction to complete paralysis is  
677 reproducible for over >10 generations of homozygous breeding. This feature contrasts with  
678 other TDP-43 mouse models reported thus far. We note that there is a dose-dependent  
679 response to increased levels of TDP-43. In some reports, excessive expression of TDP-43  
680 (e.g., >3 fold of nTg level) causes acute toxicity and rapid demise of the animals. At the other  
681 end of the spectrum, models that express too little TDP-43 provoke no phenotype or at most  
682 mild phenotypes that require careful measurements to unveil [35-42]. Few transgenic lines  
683 developed motor dysfunction and paralysis but symptoms from the digestive system, highly  
684 variant phenotypes and lifespan (e.g., ~1 to >15 months), and low penetrance complicate the  
685 phenotypes (e. g. ~5% transgenic animals) [57, 77-79]. Thus, our TDP-43 mice provide a useful  
686 model for *in vivo* study of chronic TDP-43 toxicity derived from the modest elevation of TDP-43  
687 levels and *in vivo* preclinical tests of experimental drugs targeting chronic TDP-43 toxicity in the  
688 CNS.

## 689 **Conclusions**

690 We established a transgenic mouse model with a modestly elevated TDP-43 level. This model  
691 displays several ALS characteristics, including late-onset and progressive paralytic motor  
692 dysfunction ending in paralysis, neuroinflammation, and neurodegeneration. This study  
693 demonstrates that modest elevations in TDP-43 expression can trigger neurodegeneration and  
694 clinical phenotypes of ALS, suggesting that modestly elevated TDP-43 levels in humans could  
695 cause ALS and other neuromuscular disorders involving TDP-43 proteinopathy. Because of the

696 easily observable, predictable, and progressive clinical paralytic phenotypes, this transgenic  
697 mouse model may be useful in preclinical trials of therapeutics targeting neurological disorders  
698 associated with elevated levels of TDP-43.

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- 986

987 **Supporting Information**

988 **S1 Fig. Generation of transgenic mice that overexpress wild-type TDP-43.** (A) A cDNA  
989 encoding mouse TDP-43 and EGFP linked by internal ribosome entry site (*IRES*) were inserted  
990 into the backbone of MoPrp.Xho plasmid to generate the Prp-mouse TDP-43 transgene. The  
991 construct was composed of the following elements in linear succession: the Prp promoter,  
992 mouse wild-type TDP-43, IRES, EGFP gene, and poly A signal. This construct (Prp-TDP-43)  
993 expresses TDP-43 and GFP separately. (B) Western blot showed that the transgene was  
994 expressed in all the CNS regions in transgenic lines 19 and 42. FC, frontal cortex; CSC, cervical  
995 spinal cord; LSC, lumbar spinal cord; BS, brainstem; CB, cerebellum. (C) A survey of the  
996 transgene expression in different organs in transgenic line 19 showed that the transgenes were  
997 predominately expressed in CNS. Low levels of expression were also detected in heart (Hrt),  
998 lung (Lg) and kidney (Kdn). Other tissues are muscle (Msl), liver (Lv), and spleen (Spl). (D) A  
999 survey of the transgene expression in different organs in transgenic line 42. Similar to line 19,  
1000 the transgenes were predominately expressed in the CNS. The samples in B, C, and D were  
1001 prepared from animals between 55 and 65 days old. (E) A mouse at the paralysis stage. Its  
1002 limbs were paralyzed, and the mouse lost its local motion capability. (F) Monitoring small  
1003 cohorts of mice from lines 19 and 42 showed late-onset paralysis but incomplete penetrance  
1004 from both lines up to 750 days. (G) Progressive weight loss in the aged mice of the two  
1005 transgenic lines. The animal numbers are 3 to 4 for line 42, 3 to 5 for line 19, and 3 to 15 for  
1006 non-transgenic (nTg) control mice at different age points. For the TDP-43 mice, only animals  
1007 that develop paralysis were included in the weight plots. Error bars are standard errors.

1008 **S2 Fig. Insoluble TDP-43 is increased in TDP-43 Tg mice.** (A) Western blot of detergent-  
1009 soluble and insoluble TDP-43 and ubiquitinated proteins extracted from lumbar spinal cords of  
1010 paralyzed Tg mice and age-matched nTg controls. Each lane was loaded with proteins from one  
1011 animal. Arrows point to TDP-43 and its 35 KD and 25 KD fragments. Numbers on the right

1012 indicate molecular weights in kD. (B) Relative ratios of band intensity of the pellet over the  
1013 supernatant. Bars represent averages of 7 nTg and 8 Tg animals in the TDP-43 quantification  
1014 and of 4 animals in both nTg and Tg groups in the ubiquitin quantification. Student t test was  
1015 used to compare between Tg and nTg mice. \* indicates  $p < 0.05$  and \*\*\*  $p < 0.001$ .

1016 **S3 Fig. Widespread demyelination in the spinal cord of the Tg mice.** (Aa, e) cortical spinal  
1017 track, (Ab, f) lateral funiculus, (Ac, g) ventral funiculus, (Ad, h) anterior commissure. (B) Electron  
1018 microscopic images of ventral funiculus from a nTg mouse (left) and a Tg mouse (middle and  
1019 right). Notice very few axons were wrapped by myelin in the Tg mice.

1020 **S4 Fig. Inflammation markers that did not significantly change from the levels of nTg**  
1021 **mice.** (A) cyclooxygenase 1 (COX-1 or PTGS1). (B) lipocalin type of prostaglandin synthase  
1022 (LPGDS). (C) prostaglandin D2 receptor (DP1). (D) prostaglandin D2 receptor 2 (DP2). (E)  
1023 inducible nitric oxide synthase (iNOS). (G) neuronal nitric oxide synthase (nNOS). Student t  
1024 tests with Bonferroni correction were used to compare between the Tg and nTg mice at different  
1025 ages.  $n = 3-8$  at each age for both groups. No significance was found ( $p > 0.05$ ).

1026 **S5 Fig. No difference in the brain weight between the Tg and nTg mice** ( $n = 6$  in both  
1027 groups).

1028 **S6 Fig. Visualization and quantification of CTIP2-positive neurons in the motor cortex.** (A)  
1029 CTIP2 staining of the motor cortex in nTg and paralyzed Tg mice. The boxed areas in layer V  
1030 are enlarged in the two panels on the right. Notice a substantial reduction in the CTIP2 staining  
1031 intensity in the Tg mice. (B) quantification of CTIP2-positive cells in layer V. (C) Protein blot of  
1032 CTIP2 in the motor cortex from nTg and Tg mice at different ages. P-value was derived from  
1033 Student's t test.  $n = 4$  for nTg and 3 for Tg mice.

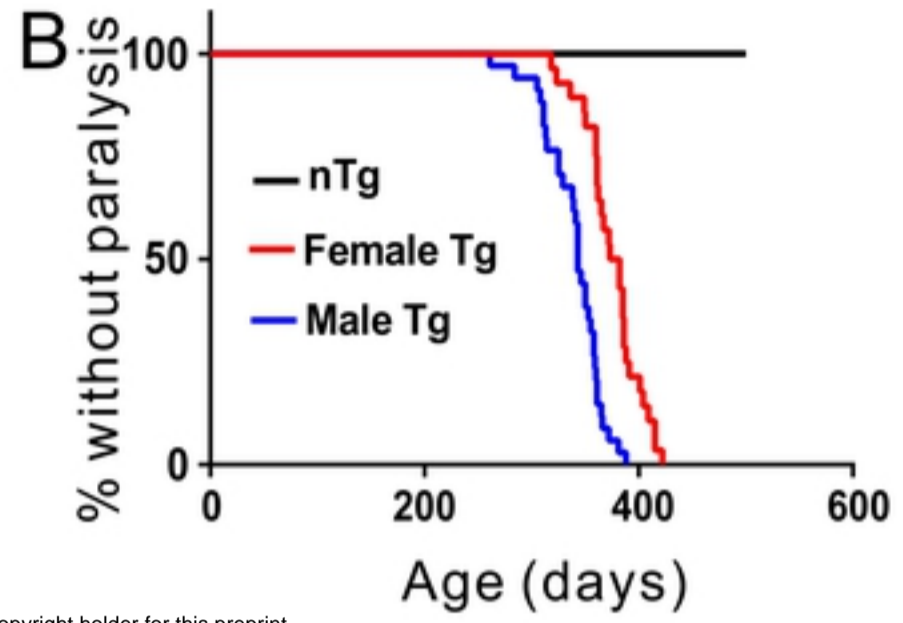
1034 **S1 Table. PCR primers for data shown in Figure 2D**

1035 **S1 Video. Tg mice developed progressive motor dysfunction and paralysis.** The  
1036 transgenic mouse was marked with red ink on its tail. The other mouse in this video was a  
1037 control nTg mouse. Notice the Tg mouse goes through stages from hyperactive to final  
1038 paralysis.

1039

1040

Figure 1



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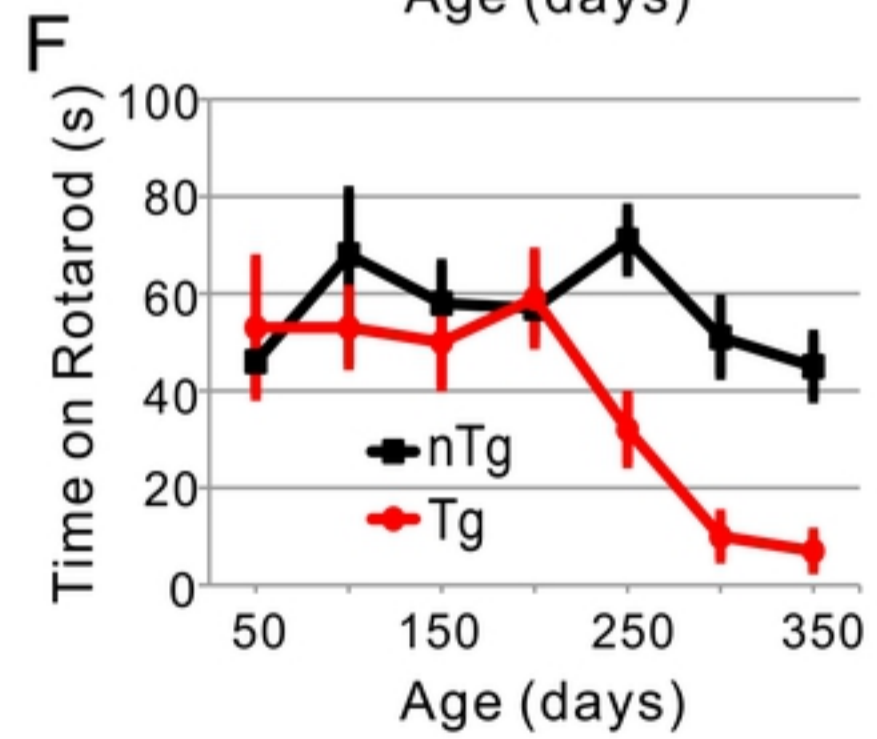
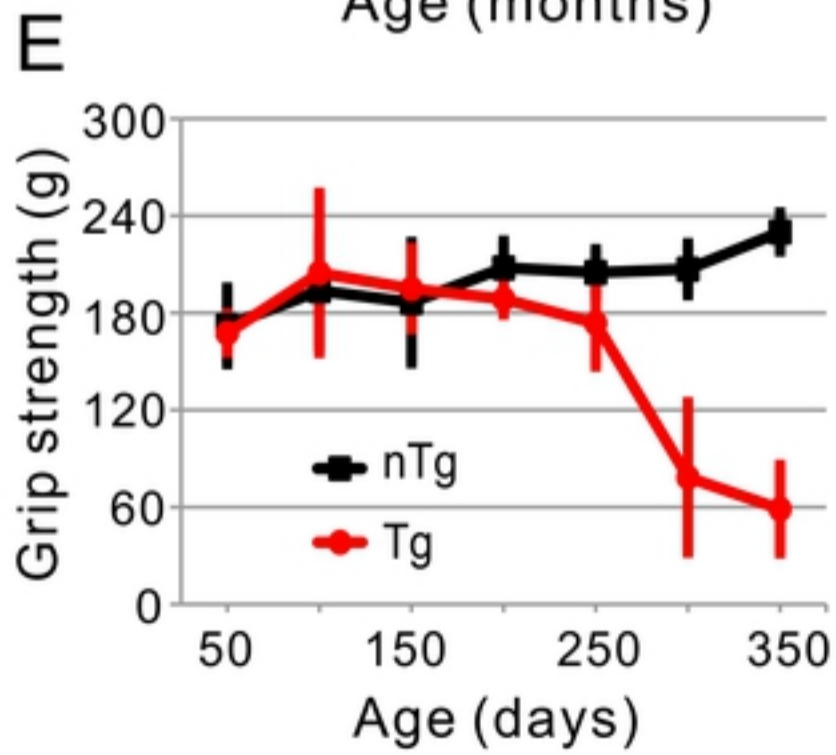
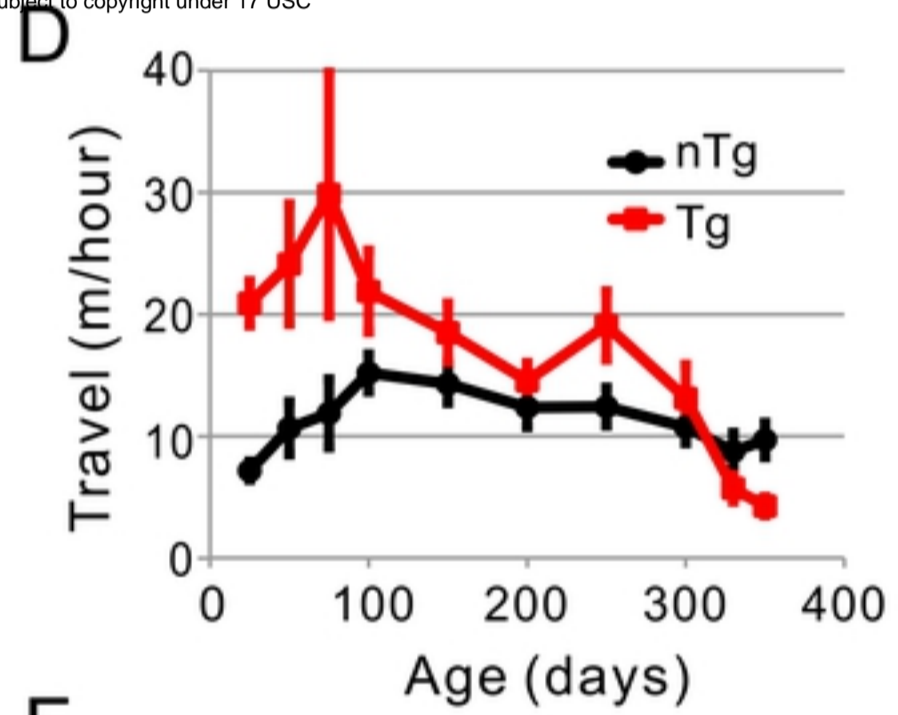
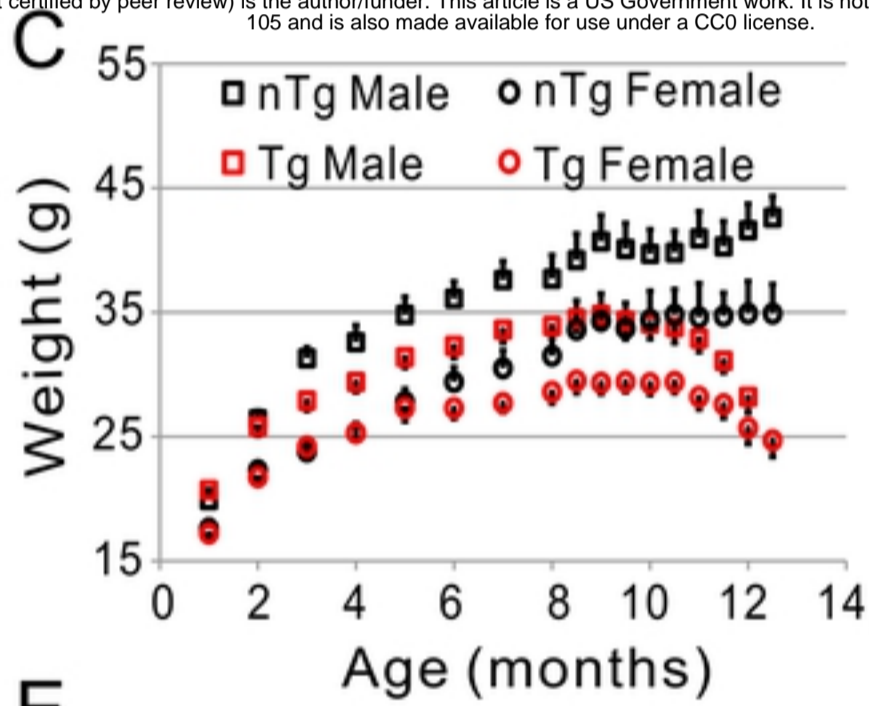


Figure 1

Figure 2

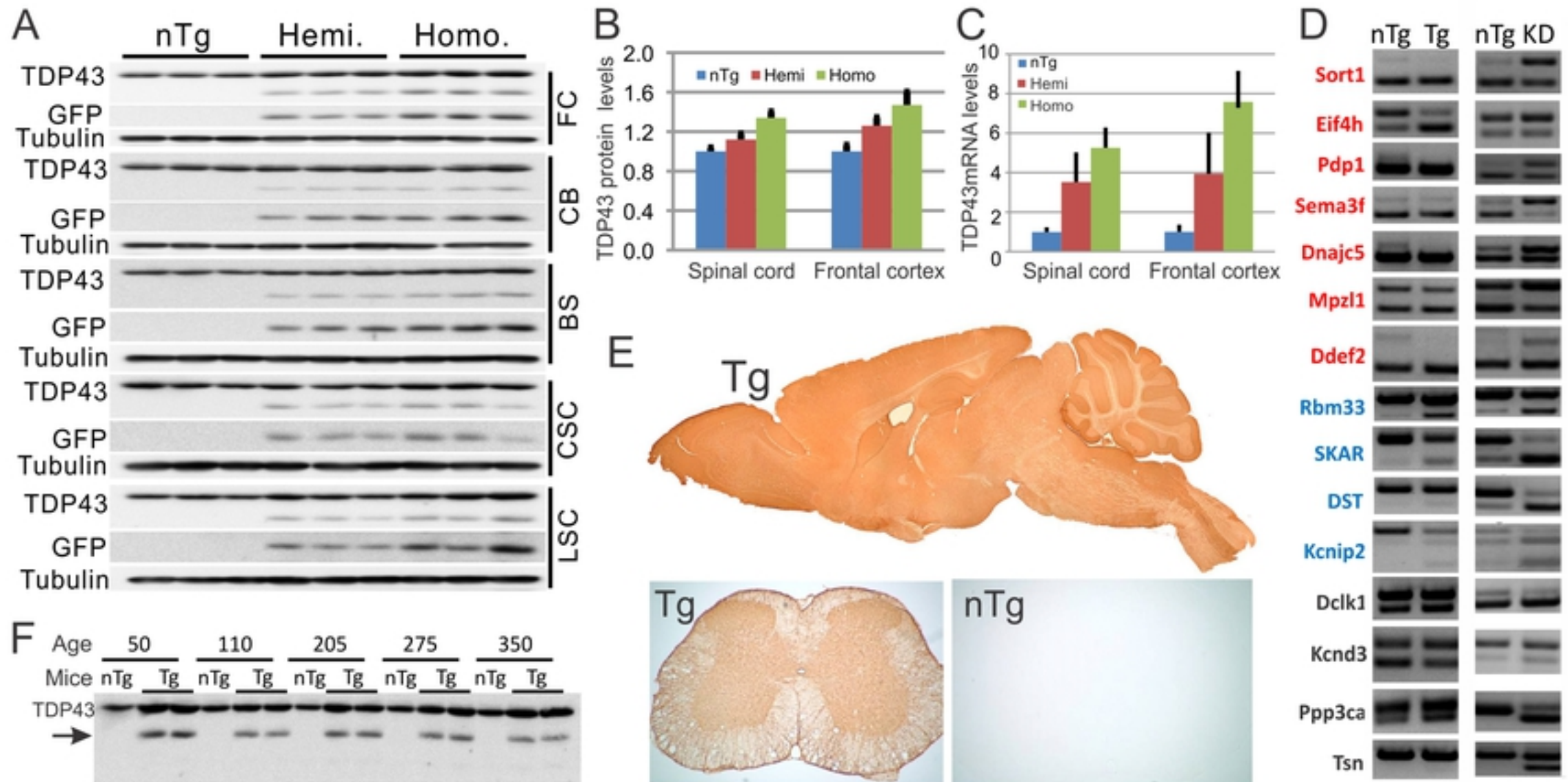


Figure 2



Figure 3

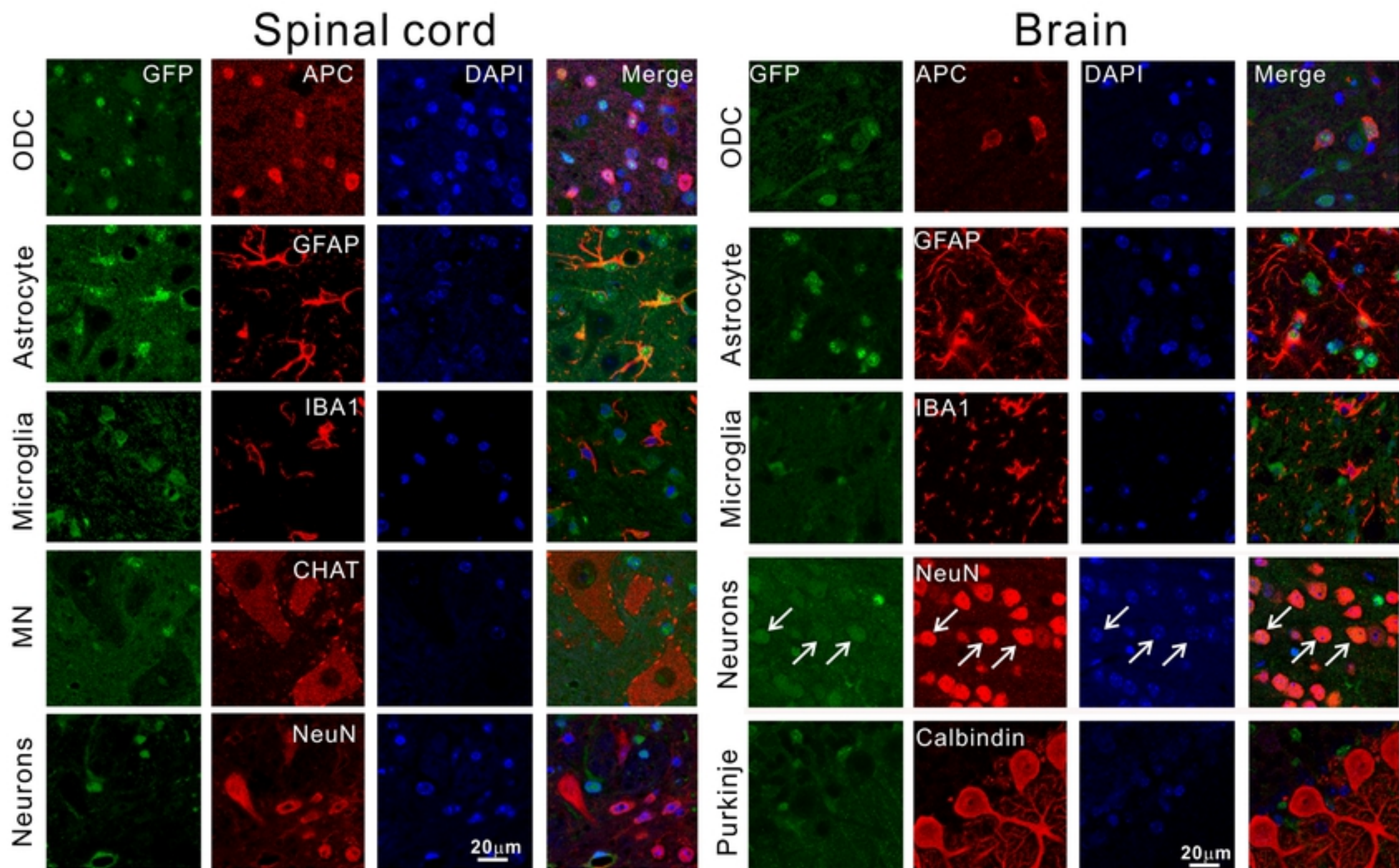


Figure 3

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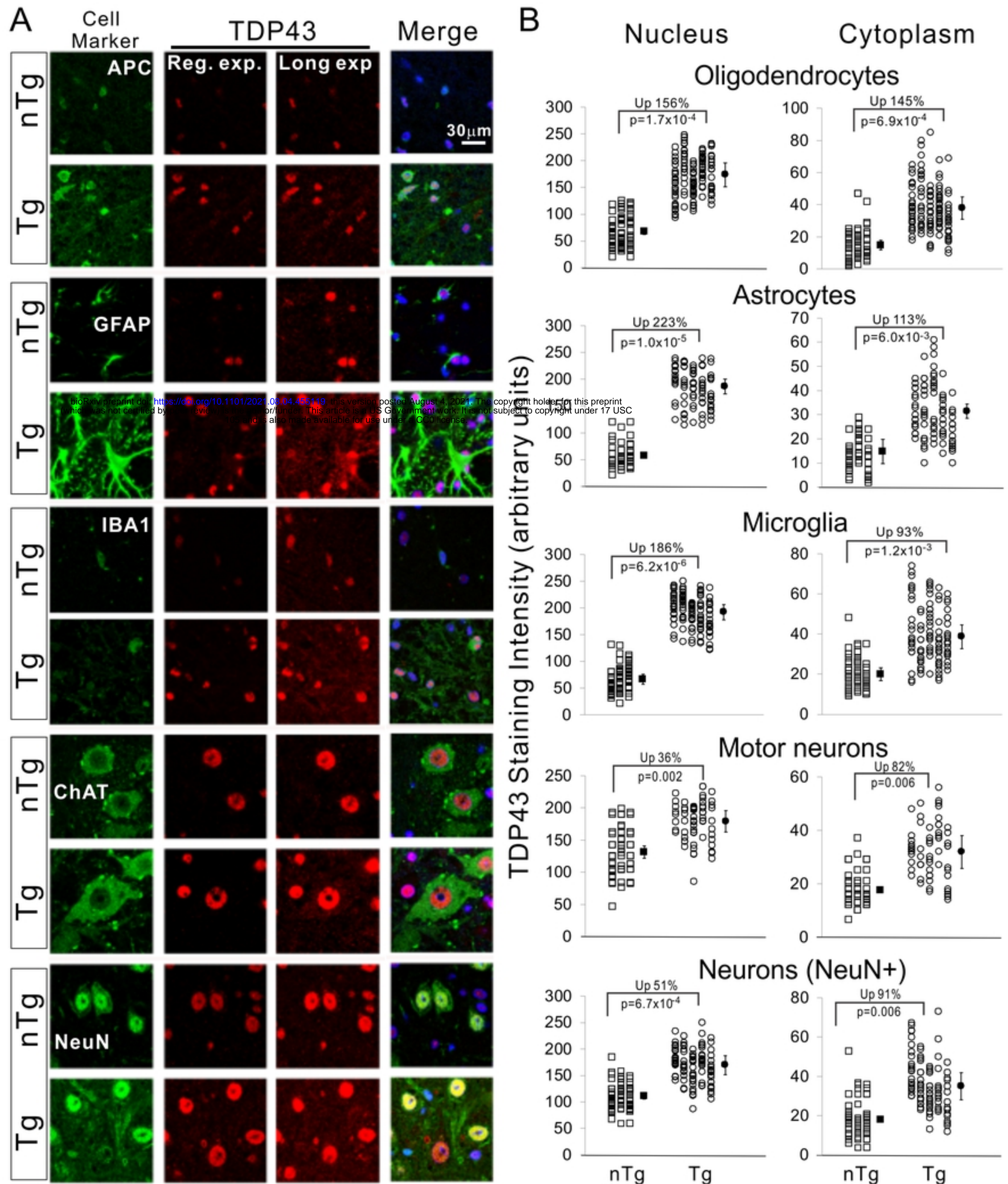


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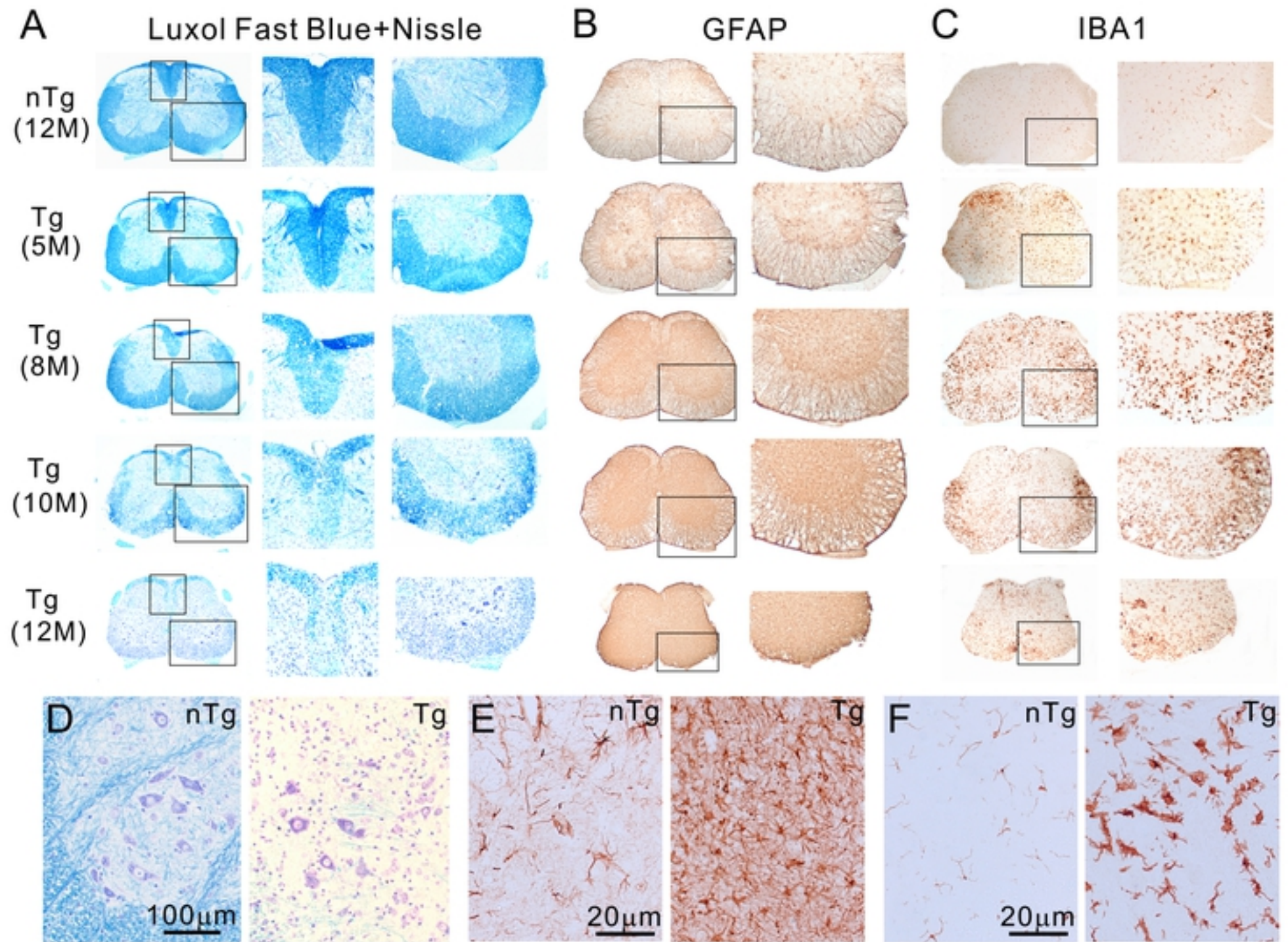


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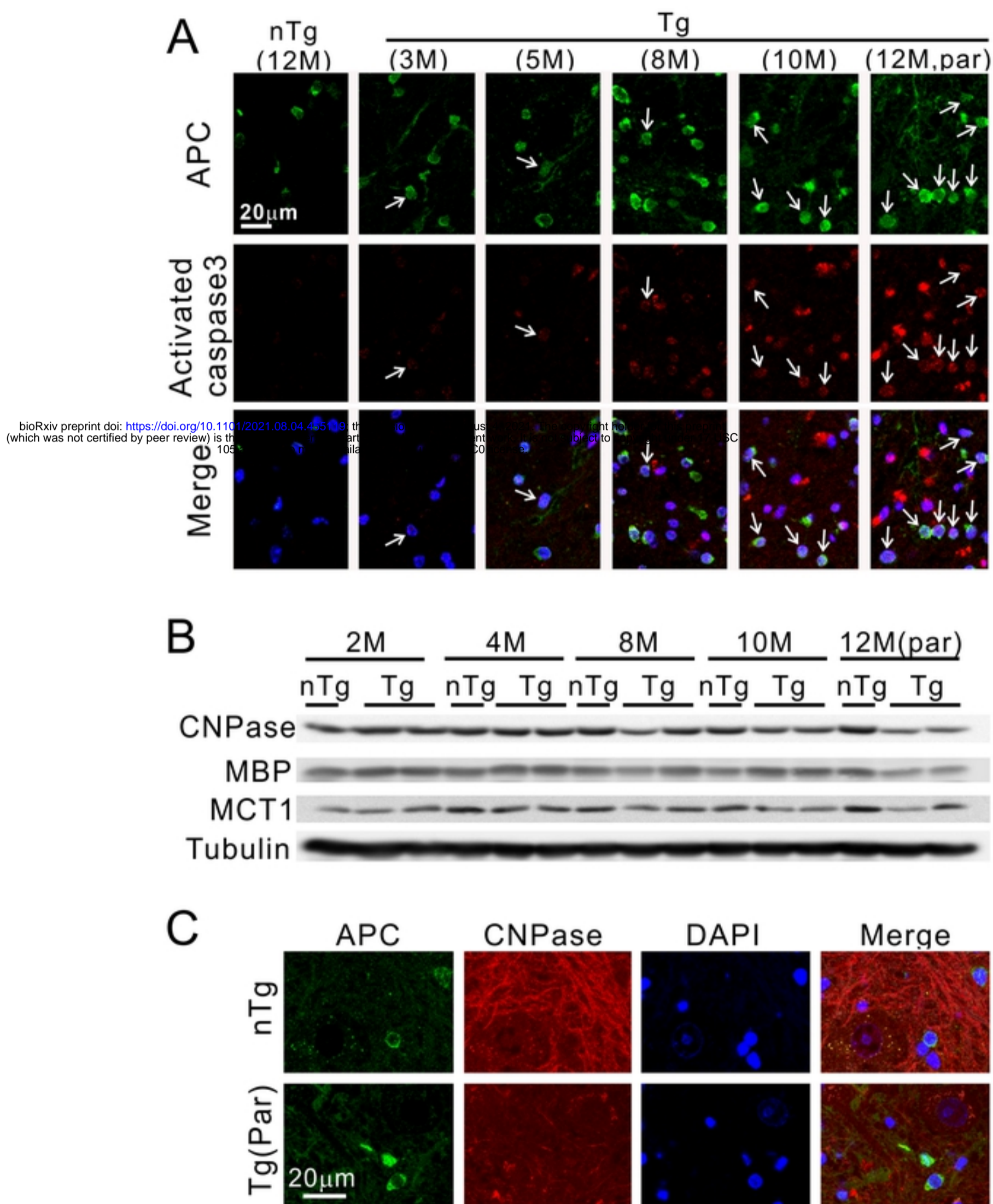


Figure 6

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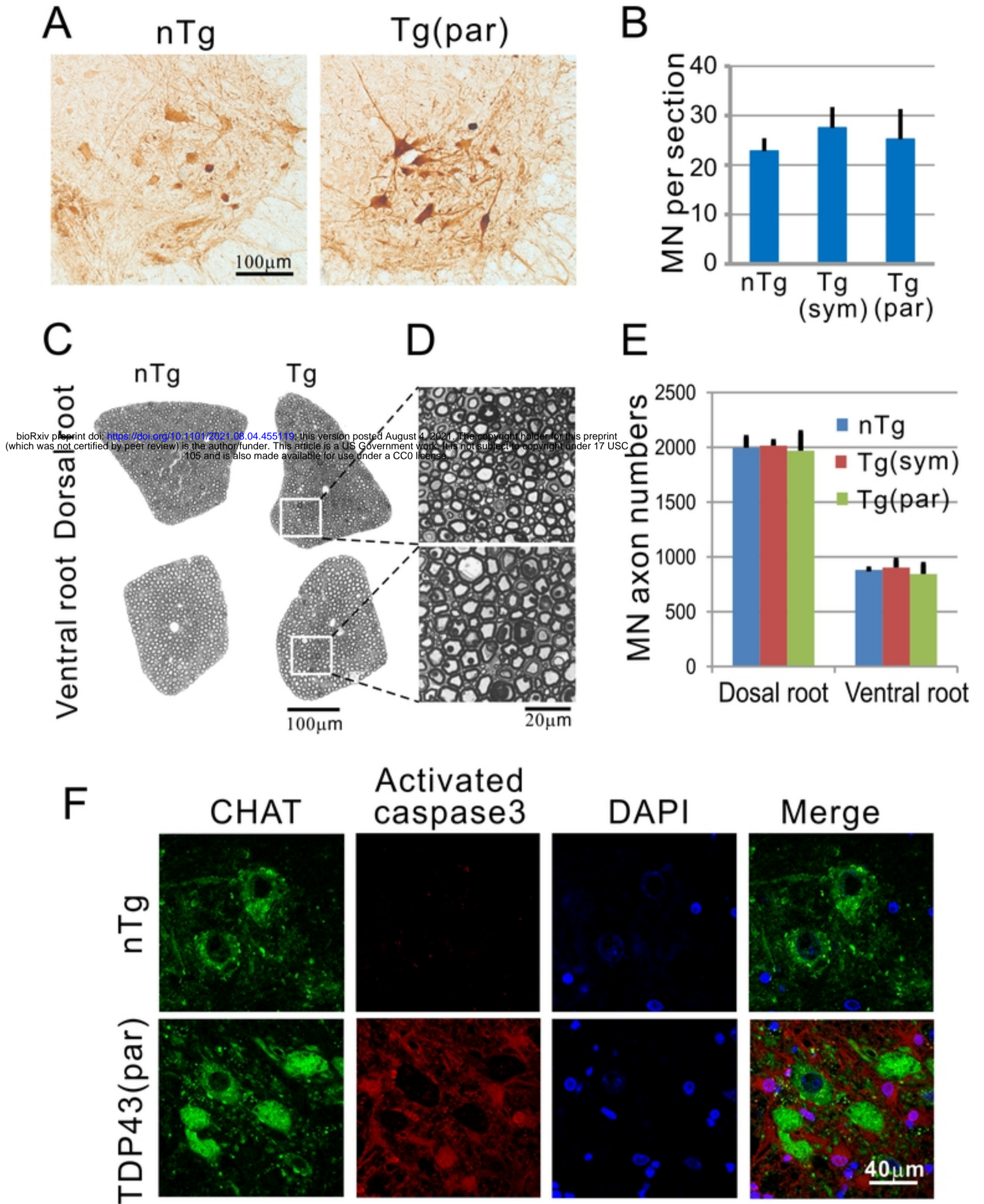


Figure 7

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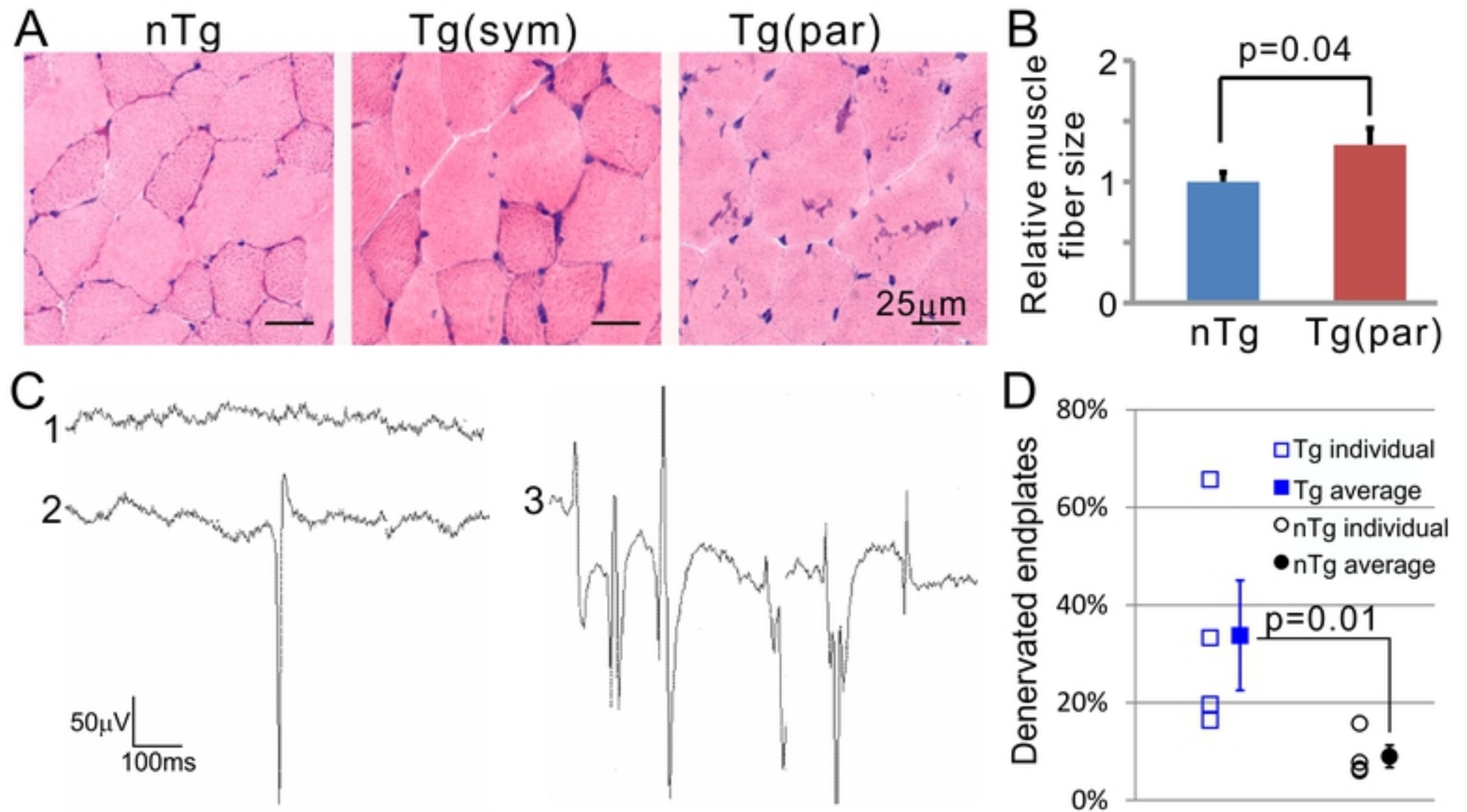


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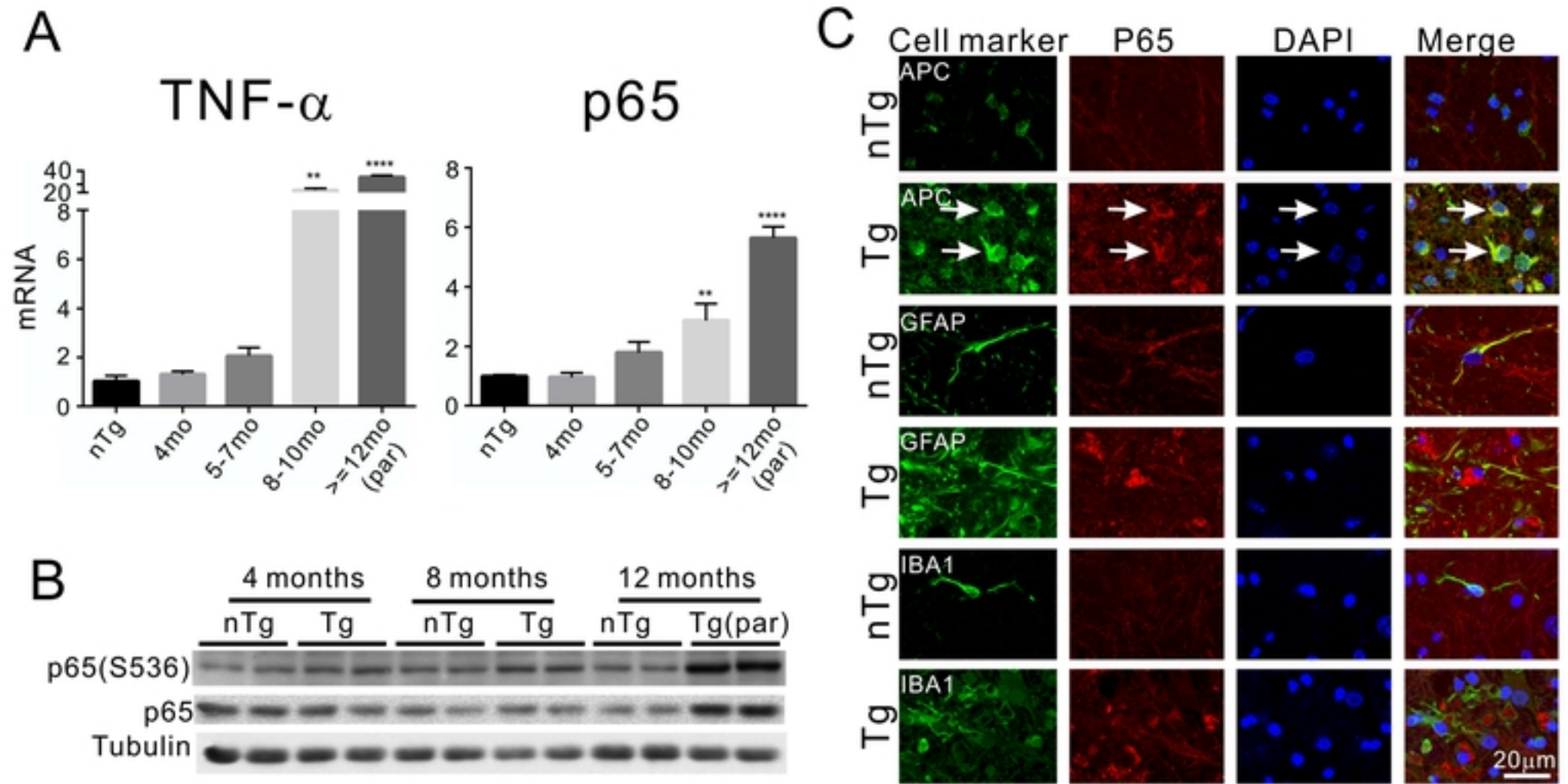


Figure 9

Figure 10

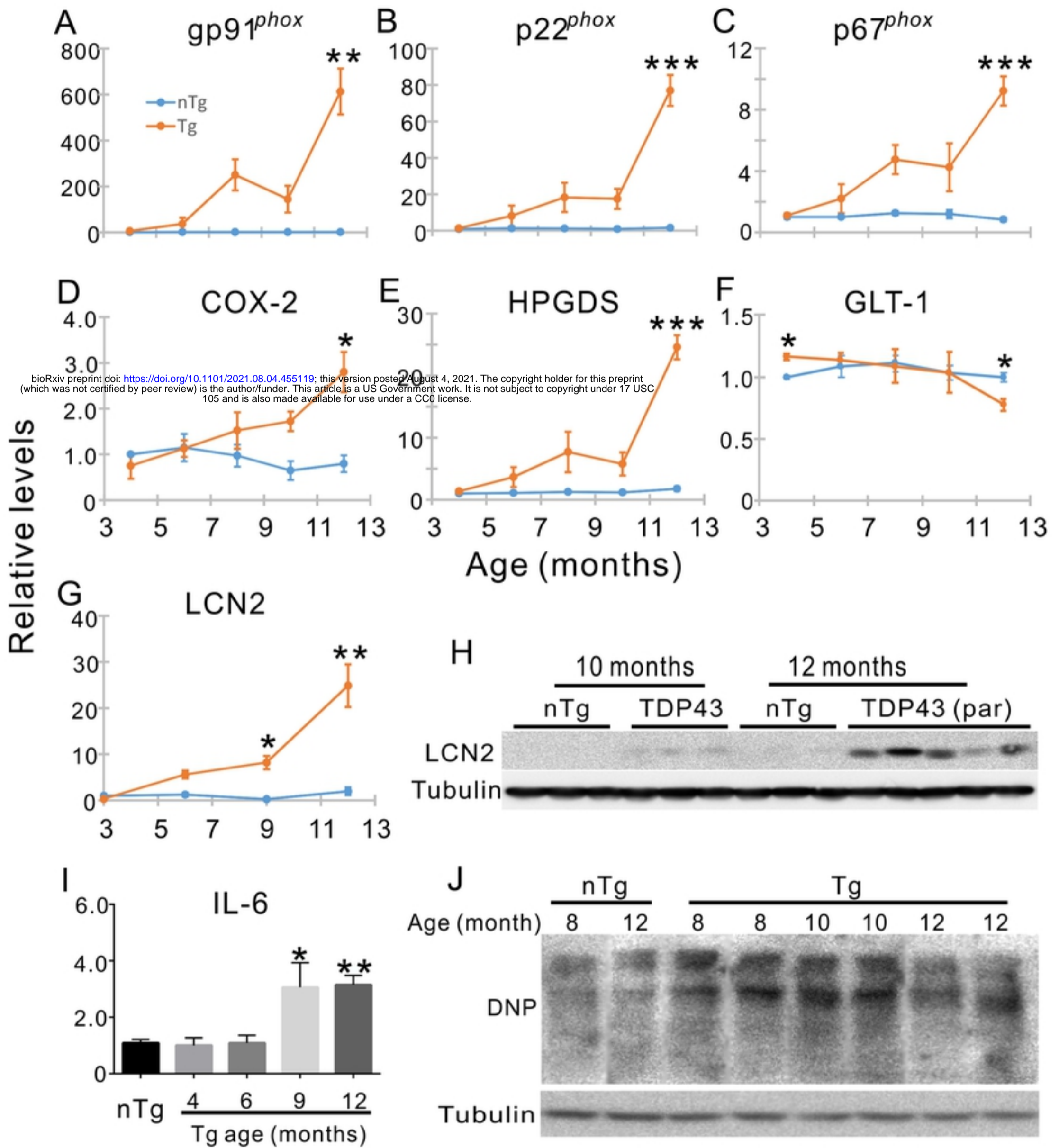


Figure 10



Figure 11

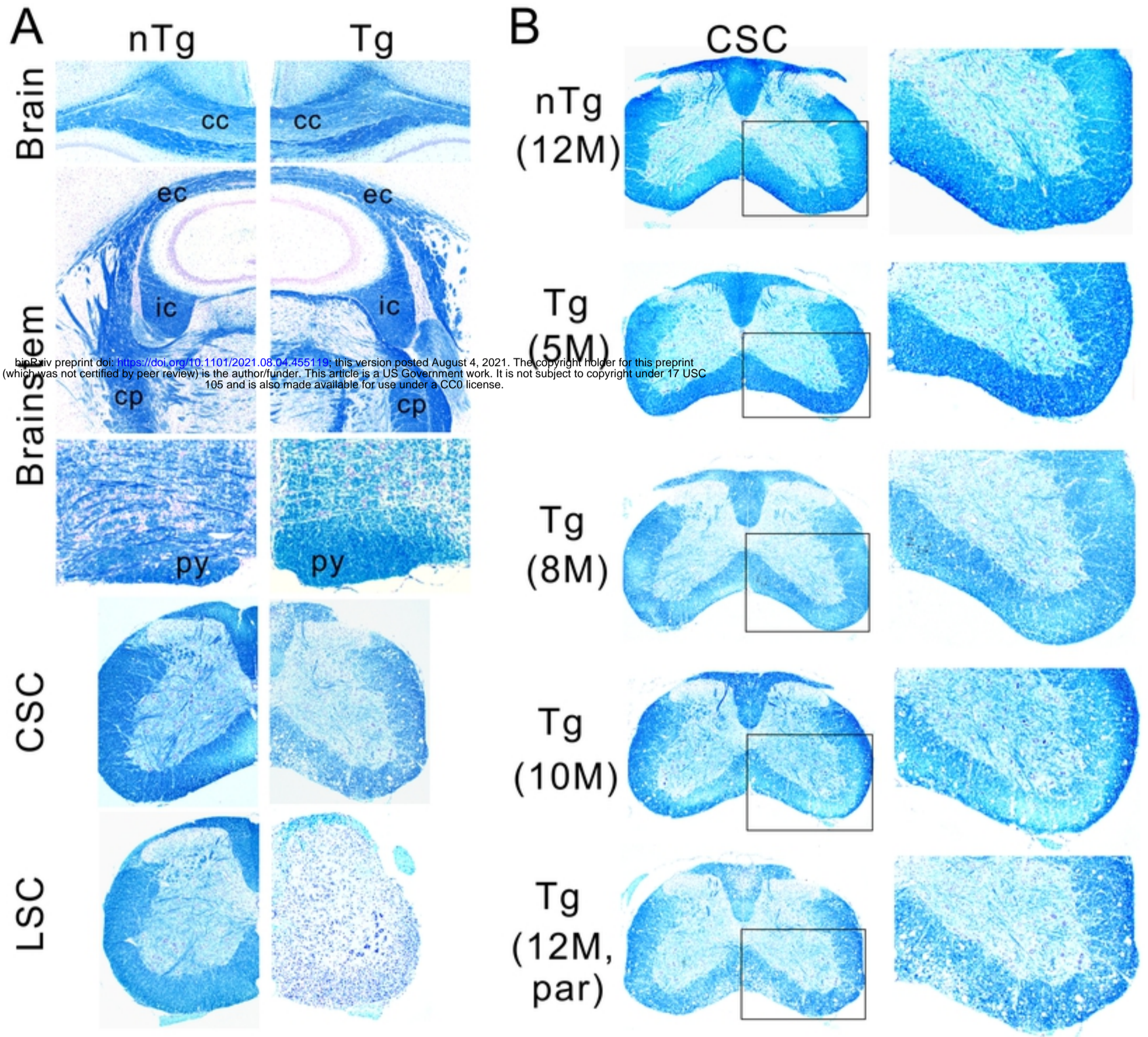


Figure 11

Figure 12

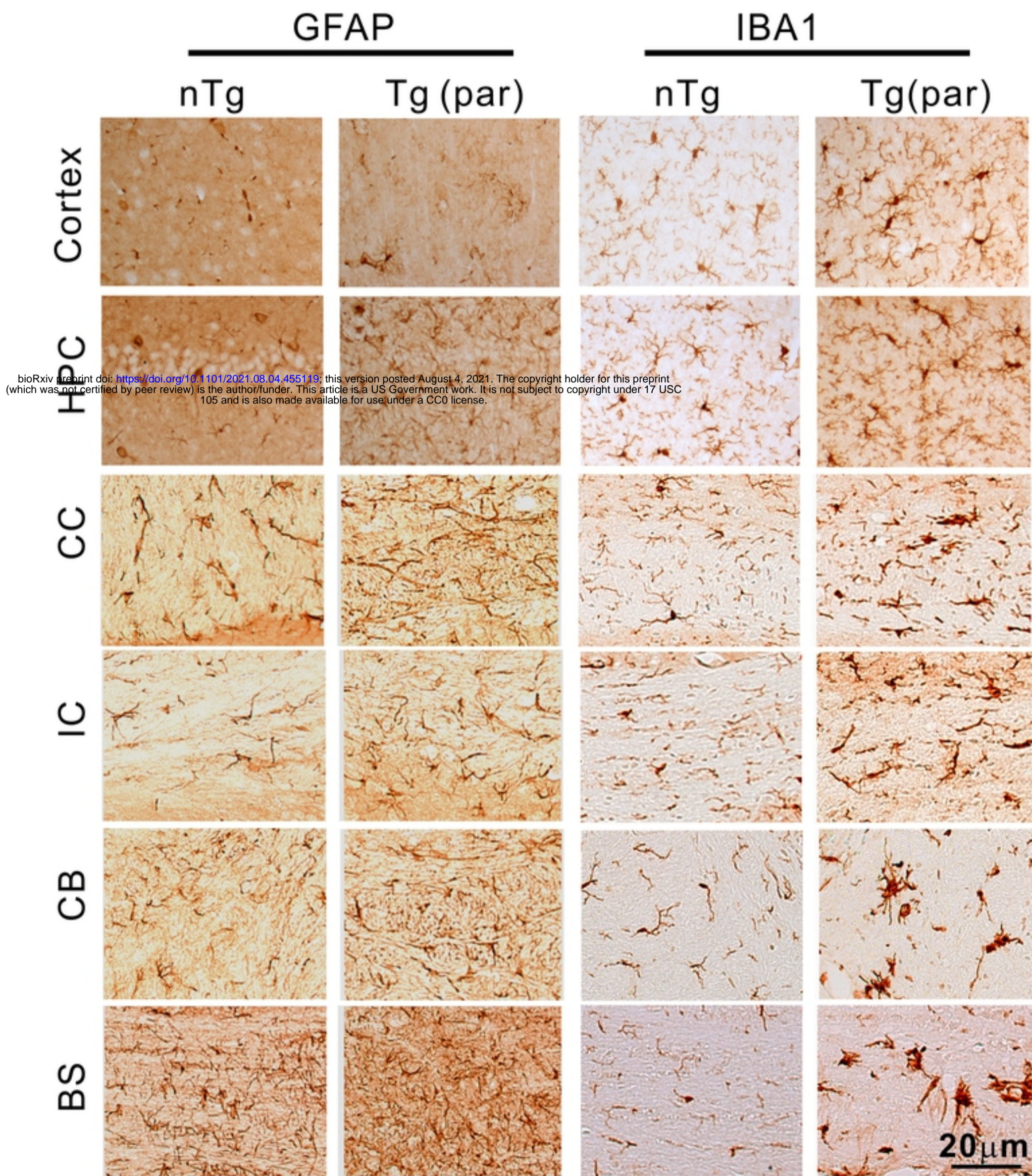


Figure 12

Figure 13

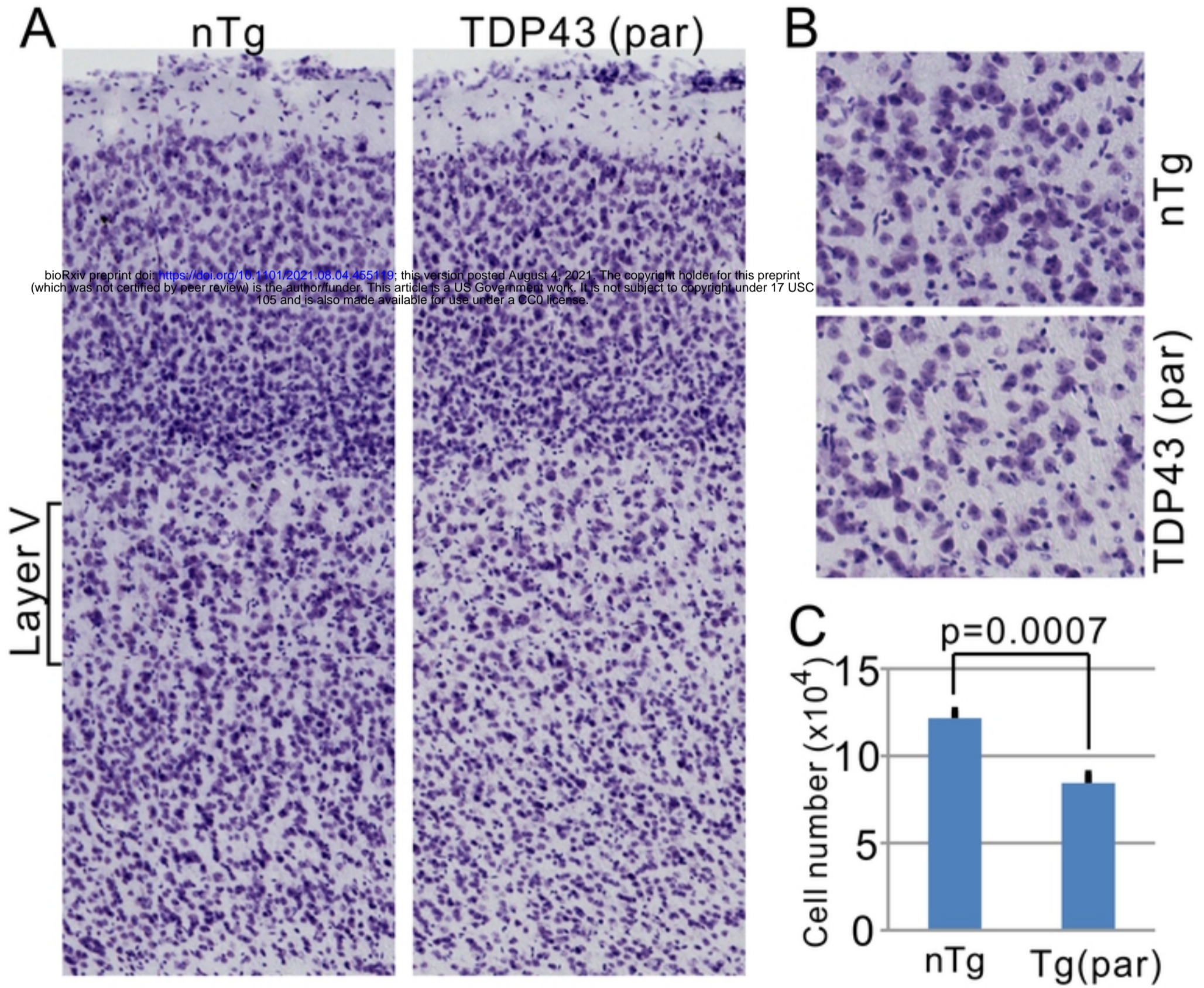


Figure 13