Targeted overexpression of Tumor Necrosis Factor-α increases Cyclin-dependent kinase 5 activity and TRPV1-dependent Ca2+ influx in trigeminal neurons

Article *in* Pain · February 2016

DOI: 10.1097/j.pain.0000000000000527

10 authors, including:

Pablo Lazcano

University of Chile

1 PUBLICATION **0** CITATIONS

SEE PROFILE

María Pertusa

University of Santiago, Chile

19 PUBLICATIONS **325** CITATIONS

SEE PROFILE

Christian González-Billault

University of Chile

81 PUBLICATIONS **1,678** CITATIONS

SEE PROFILE

Elias Utreras

University of Chile

28 PUBLICATIONS **289** CITATIONS

SEE PROFILE

Research Paper

PAIN®

Targeted overexpression of tumor necrosis factor-α increases cyclin-dependent kinase 5 activity and TRPV1-dependent Ca^{2+} influx in trigeminal neurons

Pablo Rozas[a,b], Pablo Lazcano[a,b], Ricardo Piña[c], Andrew Cho[d], Anita Terse[d], Maria Pertusa[c], Rodolfo Madrid[c], Christian Gonzalez-Billault[b], Ashok B. Kulkarni[d], Elias Utreras[a,*]

Abstract

We reported earlier that TNF-α, a proinflammatory cytokine implicated in many inflammatory disorders causing orofacial pain, increases the activity of Cdk5, a key kinase involved in brain development and function and recently found to be involved in pain signaling. To investigate a potential mechanism underlying inflammatory pain in trigeminal ganglia (TGs), we engineered a transgenic mouse model (TNF^{glo}) that can conditionally overexpresses TNF-α upon genomic recombination by Cre recombinase. TNF^{glo} mice were bred with Nav1.8-Cre mouse line that expresses the Cre recombinase in sensory neurons to obtain TNF-α:Nav1.8-Cre (TNF-α cTg) mice. Although TNF-α cTg mice appeared normal without any gross phenotype, they displayed a significant increase in TNF-α levels after activation of NFκB signaling in the TG. IL-6 and MCP-1 levels were also increased along with intense immunostaining for Iba1 and GFAP in TG, indicating the presence of infiltrating macrophages and the activation of satellite glial cells. TNF-α cTg mice displayed increased trigeminal Cdk5 activity, and this increase was associated with elevated levels of phospho-T407-TRPV1 and capsaicin-evocated Ca^{2+} influx in cultured trigeminal neurons. Remarkably, this effect was prevented by roscovitine, an inhibitor of Cdk5, which suggests that TNF-α overexpression induced sensitization of the TRPV1 channel. Furthermore, TNF-α cTg mice displayed more aversive behavior to noxious thermal stimulation (45°C) of the face in an operant pain assessment device as compared with control mice. In summary, TNF-α overexpression in the sensory neurons of TNF-α cTg mice results in inflammatory sensitization and increased Cdk5 activity; therefore, this mouse model would be valuable for investigating the mechanism of TNF-α involved in orofacial pain.

Keywords: Orofacial pain, Inflammation, Cdk5, TNF-α, TRPV1

1. Introduction

Orofacial pain has a complex etiology and is associated with many diseases that include migraine, headaches, neuralgias, pulpitis, and temporomandibular disorders, among others.[15] The inflammation of orofacial tissue is involved in many of these pathologies. Inflammatory mediators secreted by damaged tissue and immune cells regulate many signal transduction cascades that increase the activity of certain kinases, leading to nociceptor sensitization and a consequent enhancement in pain sensation.[15,41,48] For example, we and others previously discovered that cyclin-dependent kinase 5 (Cdk5) is directly involved in pain signaling.[36,38,48,51,61]

Cdk5 is a proline-directed serine/threonine kinase that is ubiquitously expressed, whereas its activators p35 and p39 are mainly expressed in the postmitotic neurons.[8] Cdk5 plays key roles in brain development and function.[8,17,48] It phosphorylates many important target proteins that participate in the normal function of the brain and also in neurodegenerative diseases and disorders.[5,8,42,48,52] We and others reported earlier that Cdk5 kinase activity was increased in nociceptive primary afferent neurons after experimentally induced inflammation with carrageenan[35] and complete Freund adjuvant.[66] We also reported decreased responses to painful stimulation in p35-knockout mice with residual Cdk5 activity[35,38] and in Cdk5 conditional knockout mice deficient in Cdk5 in nociceptive neurons.[34] In contrast, p35-overexpressing transgenic mice, which have significantly increased Cdk5 activity, were more sensitive to painful stimulation.[35,38] Additionally, we reported that Cdk5 directly phosphorylates threonine 407 of transient receptor potential vanilloid 1 (TRPV1), an ion channel critically involved in thermal nociception and inflammatory pain.[4] Elevated Cdk5 activity results in an increased agonist-induced Ca^{2+} influx in cultured dorsal root ganglia (DRG)[34] and trigeminal ganglion (TG)[51] primary neurons, and in odontoblast-like cells.[53] Furthermore, to identify inflammatory cytokines that regulate Cdk5 activity in response to inflammation, we developed a cell-based assay that measures the expression level of p35, a limiting factor of Cdk5 activity.[46] Using this cell-based assay, we identified tumor necrosis factor-α (TNF-α) as a major regulator of Cdk5 activity.[48,49] TNF-α is a pleiotropic

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a] *Laboratory of Molecular and Cellular Mechanisms of Pain, Department of Biology, Faculty of Sciences, Universidad de Chile, Santiago, Chile,* [b] *Laboratory of Cellular and Neuronal Dynamics, Department of Biology, Faculty of Sciences, Universidad de Chile, Santiago, Chile,* [c] *Department of Biology, Faculty of Chemistry and Biology, University of Santiago de Chile, Santiago, Chile,* [d] *Functional Genomics Section, Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*

**Corresponding author. Address: Department of Biology, Faculty of Sciences, Universidad de Chile, Las Palmeras 3425, Ñuñoa 7800024, Santiago, Chile. Tel.: 562-29787423; fax: 562-22712983. E-mail address: elias.utreras@uchile.cl (E. Utreras).*

PAIN 157 (2016) 1346–1362

http://dx.doi.org/10.1097/j.pain.0000000000000527

cytokine that participates in a wide range of diverse cellular responses, including cell death, survival, activation, differentiation, and proliferation.[7] After stress, injury, or during inflammation, TNF-α is expressed and secreted by many cell types including immune cells, astrocytes, microglia, and neurons.[24,43] TNF-α exerts its biological functions through the action of TNFR1 and TNFR2, which are expressed in DRG[16] and TG[18] neurons. In rat TGs, TNF-α increases the sensitivity of TRPV1$^+$ neurons to capsaicin in part by sustained upregulation of TRPV1 expression.[18]

In this study, we investigated the regulation of Cdk5 activity by TNF-α in vivo by generating a conditional transgenic mouse that specifically overexpresses TNF-α in nociceptive neurons using the Cre-loxP recombination system. Our studies on these mice indicate that TNF-α regulates Cdk5 activity in TGs, implicating its potential role in orofacial pain.

2. Methods

2.1. Cloning of TNF-α pCLE transgenic vector

To engineer a mouse model that conditionally overexpresses TNF-α, we first generated the pCLE-TNF-α vector by subcloning mouse TNF-α cDNA into the pCLE vector.[3] The pCLE vector contains a 1.7 kb β-actin promoter combined with a CMV-IE enhancer (CAG promoter) for ubiquitous expression of the transgene, and downstream from the promoter is a 1-kb enhanced green fluorescent protein (EGFP) gene that is flanked by loxP sites. After the EGFP gene, there is a Kozak sequence (GACACC) followed by a mouse TNF-α cDNA sequence (708 bp) subcloned directionally between EcoRI and BamHI restriction sites (**Fig. 1A**). The pCLE-TNF-α vector was then transformed into DH5-α-competent E. coli cells, and positive clones were screened by polymerase chain reaction (PCR) using TNF-α primers and confirmed by DNA sequencing.

2.2. Transient transfection of PC12 cells and luciferase reporter activity assays

PC12 cells (ATCC#CRL-172) were transiently transfected with pCLE-TNF-α not only to study the efficiency of recombination-mediated expression of TNF-α but also to test its functionality of TNF-α overexpression. PC12 cells were transiently transfected with pCLE-TNF-α and CMV-Cre vector pBS185[40] by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 hours. The supernatants were collected and stored at −80°C for subsequent TNF-α enzyme linked immunosorbent assay (ELISA) assays. Protein and total RNA were obtained for Western blot and reverse transcription polymerase chain reaction (RT-PCR) analysis, respectively. We reported earlier that TNF-α treatment increases p35 promoter activity.[49,54] Therefore, to analyze whether the overexpressed TNF-α is secreted and active, we cotransfected pCLE-TNF-α and CMV-Cre vectors transiently into PC12 clone C7 (stably transfected with p35 promoter–luciferase vector) for 24 hours.[54] After that, the proteins were extracted from the treated cells, and p35 promoter-driven luciferase activity was measured using the Luciferase reporter assay system (Promega, Madison, WI). As a positive control to test for TNF-α functionality, we also transfected a CMV-TNF-α vector into clone C7, and the p35 promoter-driven luciferase activity was measured.

2.3. Generation of TNF^{glo} mice

To generate TNF^{glo} mice, we injected the pCLE-TNF-α vector into the zygotes of FVB/N mice as described previously.[6] Three TNF^{glo} founder lines (B1, B11, and C10) were identified through green fluorescent protein (GFP) visualization using the Macro Imaging System from Light Tools Research. All experimental studies and procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, NIH and the Ethics Committee of the Biology Department, Faculty of Sciences, University of Chile.

2.4. Genotyping of TNF^{glo} mice and generation of TNF-α cTg mice

The founder lines were genotyped by Southern blot analysis as described.[13] In addition, mice were genotyped using PCR with specific primers for GFP and an internal control (IC) generating a 173-bp and 324-bp product size, respectively (**Table 1**). Polymerase chain reaction for GFP was performed for 35 cycles consisting of 30 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C. TNF^{glo} mice were bred with Nav1.8-Cre mice[1] to generate TNF-α cTg mice that overexpress TNF-α specifically in nociceptive neurons. Polymerase chain reaction for Cre was performed with specific Cre primers generating a 421-bp product size (**Table 1**). Cre PCR was performed for 30 cycles consisting of 1 minute at 94°C, 30 seconds at 60°C, and 1 minute at 72°C. All PCR products were electrophoresed on 2% agarose gels in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.4) and stained with ethidium bromide (1 μg/mL).

2.5. Body weight and collection of tissues and serum from TNF-α cTg and control mice

All mice were killed with a lethal dose of 10% ketamine–2% xylazine (100 μL/each) injected intraperitoneally. TNF-α cTg and control mice were weighed every 2 days, starting at weaning day (P21) until 3 months of age. Body weight gain was plotted as grams vs age, and the mean values were compared between the 2 genotypes and sexes. Blood samples were collected by cardiac puncture in 1- and 3-month-old mice. Blood samples were incubated for 1 hour at room temperature followed by 5 minutes on ice and centrifuged at 3500 rpm for 20 minutes at 4°C, and supernatants (serum) were stored at −80°C for subsequent TNF-α ELISA assays (Invitrogen #KMC3012) and Cytometric Bead Array (CBA, BD#552364). Additionally, TG, brain, and spleen tissues were collected to obtain proteins and total RNA that were stored at −20°C for further analysis.

2.6. Immunofluorescence analysis

To analyze expression and localization of molecular markers of interest, TGs from 3-month-old control and TNF-α cTg were carefully dissected under a microscope (Olympus SZ51) to maintain tissue integrity and were placed in a fixing solution (4% paraformaldehyde) for 24 hours followed by incubation in a sucrose solution (30%) for an additional 24 hours. To avoid decrease in endogenous GFP fluorescence, some TGs from control and TNF-α cTg mice were fixed for 20 minutes in Zamboni solution (4% paraformaldehyde, 0.2% picric acid, 0.1 M phosphate buffer, pH 7.4) and then incubated in 30% of sucrose solution for 24 hours. Trigeminal ganglia were then embedded in OCT (Optimal Cutting Temperature from Sakura Finetek, Tokyo, Japan) and sliced into 14-μm sections on a cryostat (Microm HM 525). For immunofluorescence, the sections were incubated with a blocking/permeabilization solution (0.3% Triton X-100, 5% bovine serum albumin [BSA]) for 1 hour at room temperature followed by incubation with primary antibody in 1% BSA in

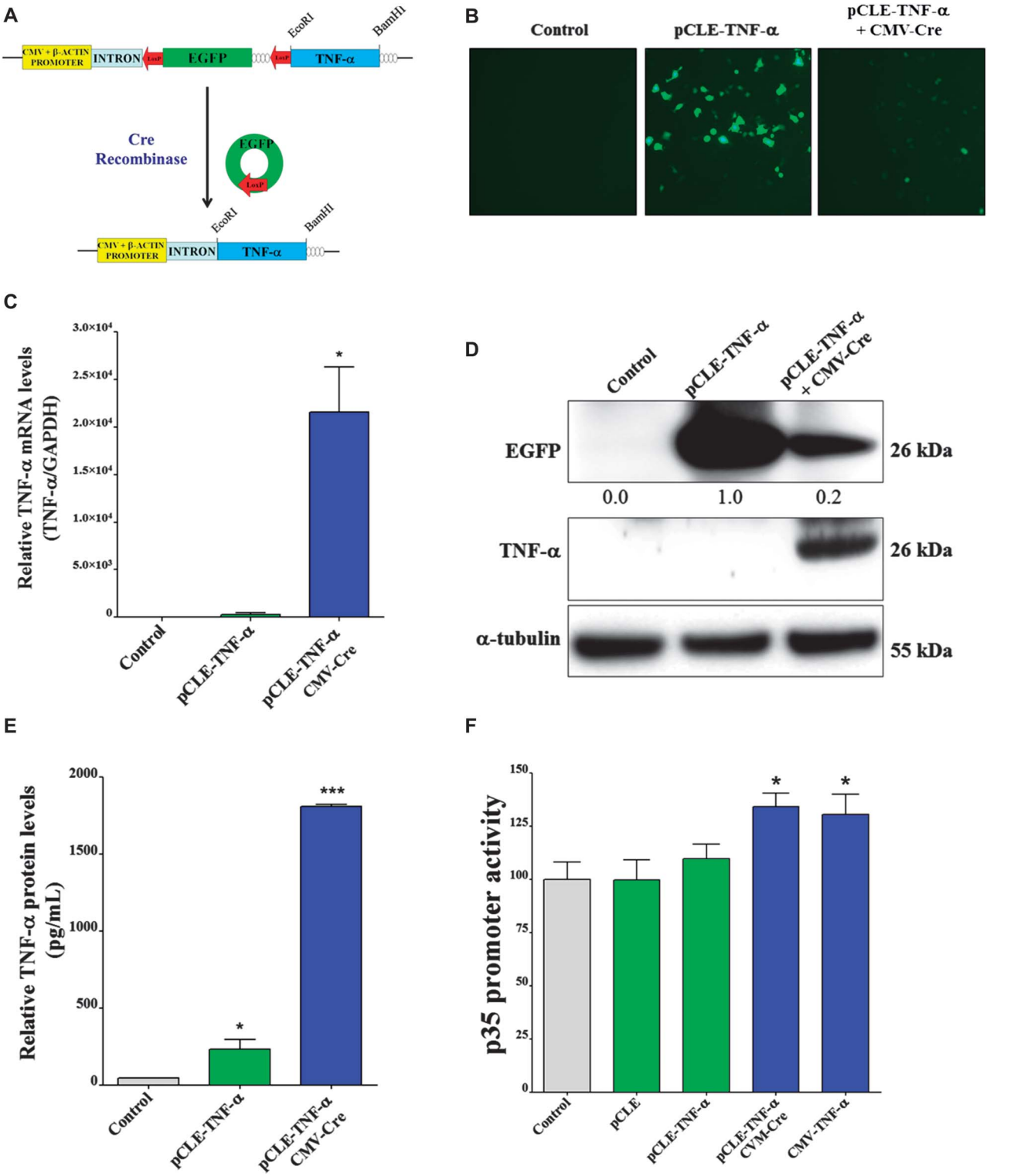

Figure 1. Cloning and characterization of pCLE-TNF-α vector. (A) pCLE-TNF-α vector was engineered by subcloning mouse TNF-α cDNA into pCLE vector.[3] pCLE vector has a global promoter for ubiquitous expression of TNF-α gene, although its transcription was interrupted by floxed EGFP gene sequence. Therefore, by using the Cre recombinase, the EGFP gene is released and the TNF-α is expressed. (B) Representative GFP fluorescence images from PC12 cells transfected with pCLE-TNF-α vector; pCLE-TNF-α and CMV-Cre vectors or control cells (untransfected) during 24 hours. (C) Relative TNF-α mRNA expression from PC12 cells transfected with pCLE-TNF-α; pCLE-TNF-α and CMV-Cre vectors and control cells (untransfected) as measured by real-time RT-PCR. S29 mRNA was used as the control. (D) Representative Western blot analysis for TNF-α, GFP, and α-tubulin protein expression from PC12 cells transfected with pCLE-TNF-α; pCLE-TNF-α and CMV-Cre vectors and control cells (untransfected) during 24 hours. Relative intensity of the GFP band is expressed in numbers. (E) Relative TNF-α levels in supernatant from PC12 cells transfected with pCLE-TNF-α; pCLE-TNF-α and CMV-Cre vectors and control cells (untransfected) during 24 hours measured by TNF-α enzyme linked immunosorbent assay. (F) Luciferase assay in PC12 clone C7 stably transfected with p35 promoter–luciferase vector. C7 cells were transiently transfected with: pCLE empty; pCLE-TNF-α; CMV-TNF-α (positive control); pCLE-TNF-α and CMV-Cre vectors or control cells (untransfected) and during 24 hours and p35 promoter–luciferase activity was measured by a luciferase kit. The bar graphs represent mean ± SEM (n = 3-6 samples). $^{*}P < 0.05$ and $^{***}P < 0.005$ as compared with no transfection.

Table 1

Sequence of primers used for genotyping and reverse transcription polymerase chain reaction.

Target	Primer sequences	Amplicon size (bp)
GFP	F: 5′-AAATTCATCTGCACCACCG-3′	173
	R: 5′-TCCTTGAAGAAGATGGTGCG-3′	
IC	F: 5′-CTAGGCCACAGAATTGAAAGATCT-3′	324
	R: 5′-GTAGGTGGAAATTCTAGCATCATCC-3′	
Cre	F: 5′-GCACTGATTTCGACCAGGTT-3′	421
	R: 5′-GAGTCATCCTTAGCGCCGTA-3′	
TNF-α	F: 5′-GATCTCAAAGACAACCAACTAGT-3′	255
	R: 5′-CTCCAGCTGGAAGACTCCTCCCAG-3′	
IL-12p40	F: 5′-GACATCATCAAACCAGACCCG-3′	209
	R: 5′-TTCTCTACGAGGAACGCACC-3′	
IFN-γ	F: 5′-CGGCACAGTCATTGAAAGCC-3′	119
	R: 5′-TGTCACCATCCTTTTGCCAGT-3′	
MCP-1	F: 5′-TCACCTGCTGCTACTCATTCACCA-3′	250
	R: 5′-AAAGGTGCTGAAGACCCTAGGGCA-3′	
IL-6	F: 5′-TCCTCTCTGCAAGAGACTTCC-3′	546
	R: 5′-GCCACTCCTTCTGTGACTCC-3′	
IL-1β	F: 5′-CAACCAACAAGTGATATTCTCCATG-3′	152
	R: 5′-GATCCACACTCTCCAGCTGCA-3′	
IL-10	F: 5′-ACTGGCATGAGGATCAGCAG-3′	351
	R: 5′-GAGAAATCGATGACAGCGCC-3′	
TGF-β1	F: 5′-GCAGTGGCTGAACCAAGGAG-3′	119
	R: 5′-CCCGACGTTTGGGGCTGATC-3′	
LIF	F: 5′-ACGGCAACCTCATGAACCA-3′	103
	R: 5′-GGAAACGGCTCCCCTTGA-3′	
OSM	F: 5′-TGTGGCTTTCTCTGGGGATAC-3′	230
	R: 5′-GAAGGTCTGATTTTGCGGGAT-3′	
S29	F: 5′-GGAGTCACCCACGGAAGTTCGG-3′	108
	R: 5′-GGAAGCACTGGCGGCACATG-3′	

phosphate-buffered saline overnight at 4°C. Trigeminal ganglion sections were then incubated with secondary antibodies (anti-rabbit, anti-mouse, and anti-donkey) conjugated to Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647 (Molecular Probes; Life Technologies, Grand Island, NY) in combination with TO-PRO-3 iodide (nuclear stain) for 1 hour at room temperature. Finally, TG sections were mounted in FluorSave (Merck Millipore, Darmstadt, Germany) and observed using confocal microscopy (LSM 510 and 710 Meta Model; Carl Zeiss Microscopy) and processed with LSM Image Browser (Carl Zeiss Microscopy, Jena, Germany) software. Immunofluorescence of TG sections and cultured trigeminal neurons were performed using the following antibodies: anti-βIII tubulin mouse antibody clone G7121 from Promega as neuronal markers, ionized calcium-binding adapter molecule 1 (Iba1) rabbit antibody #019-19741 from Wako (Richmond, VA) as the macrophage marker, glial fibrillary acidic protein (GFAP) mouse antibody #G3893, and glutamine synthetase (GS) rabbit antibody #G2781 from Sigma-Aldrich (St. Louis, MO) as satellite glial cell markers, phospho-p44/p42 MAPK (ERK1/2) rabbit antibody #9101, phospho-NFκB p65 rabbit antibody (Ser 468) #3039S, p35/p25 rabbit (C64B10) antibody #2680, anti-Egr1 rabbit antibody #4153S from Cell Signaling (Danvers, MA), anti-interleukin-6 (IL-6) rabbit antibody #407670 from Calbiochem (Darmstadt, Germany), anti TNF-α rabbit antibody #AB34674 from Abcam, anti-Cdk5 rabbit antibody C8, anti-TRPV1 rabbit antibody R-130, and anti-TRPA1 rabbit antibody from Santa Cruz Biotechnology (Dallas, TX). Anti-CGRP rabbit antibody was kindly provided by Mike Iadarola, NIH. Phospho-T407-TRPV1 rabbit antibody has been described earlier.[34] Isolectin B4 FITC conjugate (IB4) #L21895 from Sigma-Aldrich was used as nonpeptidergic neurons marker. Immunofluorescence was observed in LSM 510 and 710 Meta Confocal Microscope (Carl Zeiss Microscopy), and all parameters such as laser intensity and detector gain remained the same for all the experimental conditions analyzed. Immunofluorescence images were processed with LSM Image Browser software (Carl Zeiss Microscopy) and ImageJ software (NIH, Bethesda, MD). Fluorescence intensity was measured in more than 20 neurons within each TG sections by using the ImageJ ROI manager and was calculated by subtracting the background signal measured from zones on the slide without tissues.

2.7. Preparation of RNA and reverse transcription polymerase chain reaction

Conventional RT-PCR was performed as described previously.[53] Briefly, total RNA was obtained from the TG and brain of 1- and 3-month-old TNF-α cTg and control littermate mice. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. After TURBO DNA-freeTM (Ambion, Austin, TX) digestion of the total RNA sample, to remove contaminating genomic DNA, oligo(dT)-primed synthesis of cDNA from 3 μg of total RNA was made using Super-ScriptTM III reverse transcriptase (Invitrogen). We analyzed the mRNA expression of TNF-α, interleukin 12 (IL-12), interferon γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), IL-6, interleukin 1β (IL-1β), interleukin 10 (IL-10), transforming growth factor-β1 (TGF-β1), leukemia inhibitory factor (LIF), and oncostatin (OSM). **Table 1** lists primers used for RT-PCR. S29 primers were used to amplify 40S ribosomal protein S29 as a housekeeping gene.

2.8. Western blot analysis

Western blot analyses were performed as previously reported.[50] Briefly, protein extracts were obtained from the TG and brain of 1- and 3-month-old TNF-α cTg and control mice using T-PER buffer (Pierce, Rockford, IL) with Complete Mini protease inhibitor cocktail tablets and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostic, Indianapolis, IN). Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Proteins were separated in SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were soaked in blocking buffer (5% nonfat dry milk in PBS with 0.05% Tween-20 [PBST]) for 1 hour at room temperature, and then membranes were incubated overnight at 4°C with the appropriate primary antibody (see below) diluted in blocking buffer. The membranes were washed in PBST and incubated for 1 hour at room temperature with the secondary antibodies diluted in blocking buffer. Immunoreactivity was detected using the Super-Signal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). For semiquantitative analysis, nitrocellulose membranes were stripped and incubated with α-tubulin antibodies to normalize for protein loading. The optical densities of the bands were quantified using an image analysis system with ImageJ 1.46r software (NIH). Western blot from TG tissues were performed for Cdk5 using rabbit antibody C8 (Santa Cruz Biotechnology) and p35 rabbit antibody C64B10 (Cell Signaling) and α-tubulin antibody from Sigma-Aldrich as loading control.

2.9. Cdk5 kinase activity assay

Two hundred microgram of protein from TG of 3-month-old TNF-α cTg and control mice was dissolved in T-PER buffer and immunoprecipitated using 4 μg of anti-Cdk5 (C8) antibody (Santa Cruz Biotechnology). Briefly, immunoprecipitated proteins (IPs) were washed 3 times in cold PBS, and twice in kinase buffer

(20 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, and 1 mM EDTA). Then, the IP was mixed with the kinase assay mixture (100 mM Tris-HCl [pH 7.4], 50 mM $MgCl_2$, 5 mM EDTA, and 5 mM DTT) plus 5 units of (γP^{32})-ATP, and 5 μg of histone H1 as a substrate. The kinase assays were performed for 45 minutes at 30˚C, and the kinase activity reaction was stopped by adding loading buffer and boiling for 10 minutes at 95˚C. The kinase reaction was electrophoresed on a 12% polyacrylamide gel, and then the gels were exposed to x-ray films for 1 to 3 hours at −80˚C. The incorporation of P^{32} to histone H1 was quantified by measuring band intensity using an image analysis system with ImageJ 1.46r software.

2.10. Culture of trigeminal ganglion neurons

Trigeminal neurons were cultured as described previously.[28] Briefly, mouse TG were dissected out and incubated with Collagenase XI (0.66 mg/mL) and Dispase II (3 mg/mL) (Sigma-Aldrich) in an INC-mix solution (NaCl 155 mM; K_2HPO_4 1.5 mM; HEPES 10 mM; glucose 5 mM; at pH 7,4). Enzymatic digestion was performed for 45 minutes at 37˚C in 5% CO_2, and cells were cultured in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), Pen/Strep 100 μg/mL, MEM-vit (Invitrogen), and nerve growth factor (100 ng/mL; Sigma-Aldrich). Cells were plated on 6- or 13-mm poly-L-lysine-coated glass coverslips and used after 4 hours for Ca^{2+} imaging analysis or after 18 hours for immunofluorescence analysis. In some cases, roscovitine (10 μM) was added for 4 hours before Ca^{2+} imaging experiments.

2.11. Culture and transfection of HEK-293 cells

HEK-293 cells were plated in 24-well dishes at 1×10^5 cells per well, and transiently transfected with 1 μg of the indicated DNA using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions.[37] At 48 hours after transfection, Ca^{2+} imaging analysis was performed.

2.12. Ca^{2+} imaging

Trigeminal neurons and HEK293 cells were incubated with 5 μM Fura-2 AM dissolved in standard extracellular solution and 0.02% Pluronic (Invitrogen) for 45 to 60 minutes at 37˚C. Fluorescence measurements were made with a Nikon Ti inverted microscope fitted with a cooled digital CCD camera (Orca-03G; Hamamatsu, Hamamatsu City, Japan). Fura-2 AM was excited at 340 and 380 nm with a Polychrome V monochromator (Till Photonics, Munich, Germany), and the emitted fluorescence was filtered with a 510-nm longpass filter. Three hundred forty of 380 ratios (0.5 Hz) were displayed online with HCImage software (Hamamatsu, Japan). Bath temperature was controlled with a Peltier-based computer-controlled system and sampled simultaneously with the calcium signal (see below). Bath temperature was recorded with a Physitemp BAT-12 microprobe thermometer (Physitemp Instruments, Clifton, NJ) supplemented with an IT-18 T-thermocouple and digitized with an Axon Digidata 1440A AD converter running Clampex 10 software (Molecular Devices, Sunnyvale, CA).

2.13. Control of temperature and chemical stimuli

Coverslips with cultured TG neurons were placed in a microchamber and continuously perfused with extracellular solution warmed at 34˚C ± 1˚C. Capsaicin and KCl were applied using a time-controlled semiautomatic perfusion system,[39] with the outlet located over the field of neurons, allowing us to apply substances directly on these cells while keeping precise control of the temperature and drug exposure time. The horizontal bars shown in **Figure 9** indicate the opening and closing of the valves regulating the flow of the solution perfusing the neurons in the recording bath. The basal temperature of the bath was adjusted with a computer-operated Peltier device, placed directly on neurons and controlled by a feedback device as described previously.[28] TRPV1-dependent responses in cultured TG neurons were investigated by stimulating with pulses of 20 and 200 nM capsaicin for the time indicated by the horizontal bars in the figures. Neuronal viability was assessed by depolarization-induced responses produced by a 30-mM elevation in extracellular K^+ during the time indicated in the figure at the end of the experiment. For HEK-293 cells, capsaicin was applied at 3 different concentrations (2, 20, and 200 nM) in cells transfected with TRPV1-GFP and cotransfected with pcDNA3 (control) and p35 in pcDNA3 (p35), respectively.

2.14. Orofacial pain behavior test

An orofacial pain test was performed as previously described.[2,33] Briefly, control littermates and TNF-α cTg mice that were 6 to 8 months old were trained on an operant pain assessment device (OPAD) (Stoelting, Co, Wood Dale, IL) to press their faces into Peltier devices to receive a reward; mice could access a reward bottle filled with water sweetened with sugar. The Peltier devices could be heated to aversive temperatures. Mice were fasted for 15 ± 1 hours before receiving training. First, we trained control and TNF-α mice in the OPAD apparatus for 3 to 4 times at a nonaversive temperature (33˚C). Next week, we used a 20-minute OPAD session at constant temperature 45˚C (aversive), and the number of licks, lick-to-face contact ratio, and the total water intake were compared between control and TNF-α cTg mice. In another set of experiments, a ramp of temperature was created starting with 33˚C (nonaversive temperature) during 3 minutes was followed by heating to 45˚C over 30 seconds and then keeping at 45˚C (aversive temperature) for 3 more minutes. The number of licks was recorded at 33˚C and 45˚C and averaged over 4 sessions. Paired *t* tests were also performed, and for all analyses, a *P* value less than 0.05 was considered significant.

2.15. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism software version 5.0. Significant differences between experiments were assessed by an unpaired Student *t* test in which α was set to 0.05. For comparisons between control and TNF-α cTg mice at different ages, we used a 2-way ANOVA with post hoc tests. For Ca^{2+} imaging experiments, we used the Fisher test to compare the percentage of trigeminal neurons responding to capsaicin.

3. Results

3.1. Generation of TNF^{glo} mice

To generate TNF-α cTg mouse model that overexpresses TNF-α specifically in nociceptive neurons, we engineered a pCLE-TNF-α vector as described above. The pCLE vector has a global promoter for ubiquitous expression of TNF-α, although its transcription is interrupted by floxed EGFP gene sequence. Therefore, by use of Cre recombinase, one can release the EGFP gene to express TNF-α in a cell-specific manner (**Fig. 1A**). Before

generation of this mouse model, we evaluated whether the conditional overexpression system works in cell culture. For that purpose, we first transfected pCLE-TNF-α vector alone or in combination with CMV-Cre plasmid (pBS185) into PC12 cells. We found that EGFP fluorescence was strongly detected in cells transfected with pCLE-TNF-α, whereas this expression was found to be considerably decreased in cells cotransfected with pCLE-TNF-α and CMV-Cre vectors, indicating Cre-mediated deletion of EGFP (**Fig. 1B**). TNF-α mRNA levels were also significantly increased in these cotransfected cells as compared with the cells transfected only with pCLE-TNF-α or untransfected cells, as measured by qPCR (**Fig. 1C**). Furthermore, our Western blot analyses confirmed that EGFP protein expression was higher in cells transfected with pCLE-TNF-α (relative expression = 1.0), whereas it was decreased in cells cotransfected with both vectors (relative expression = 0.2). Interestingly, by Western blotting with TNF-α-specific antibody, we found a 26-kDa band that corresponds to membrane-bound TNF-α[57] only in cells cotransfected with pCLE-TNF-α and CMV-Cre vectors as compared with the cells transfected only with pCLE-TNF-α or untransfected cells (**Fig. 1D**). However, we were unable to detect soluble TNF-α (17 kDa) in these cell extracts. However, when we analyzed secretion of TNF-α into supernatant using TNF-α ELISA analysis, we found a significant increase in secreted TNF-α in the supernatant from the cells cotransfected with both vectors as compared with the cells transfected only with the pCLE-TNF-α vector or the untransfected cells (**Fig. 1E**). Finally, to evaluate whether the secreted TNF-α was functionally active, we used the PC12 stably clone C7 that was transfected with p35 promoter–luciferase vector to determine whether the secreted TNF-α increased p35 promoter activity.[49,54] To perform this analysis, C7 cells were transiently transfected with pCLE-TNF-α and CMV-Cre vectors, and after 24 hours, p35 promoter luciferase activity was measured. We found that TNF-α overexpression significantly increased p35 promoter activity in stable clones as compared with cells transfected only with pCLE-TNF-α vector or untransfected cells (**Fig. 1F**). As a positive control, we transfected CMV-TNF-α vector that overexpressed high levels of TNF-α, as detected by the ELISA (data not shown), into C7 cells and found that p35 promoter-luciferase activity was significantly increased (**Fig. 1F**). Altogether, these results clearly demonstrate that Cre-mediated TNF-α overexpression occurred in a cell culture system, the overexpressed TNF-α was secreted into the medium, and it was functionally active. Having shown that our strategy works in the cell culture system, we microinjected linear pCLE-TNF-α vector into FVB/N zygotes to generate TNF^{glo} mice. Thirty-five pups were obtained from 3 recipient females that were transplanted with the injected zygotes. We identified 3 founder lines (B1, B11, and C10) based on visual inspection for GFP using the Macro Imaging System (**Fig. 2A**) and by PCR analysis using GFP primers (data not shown). Three founder lines were established and confirmed for integration of the pCLE-TNF-α vector using Southern blot analysis (representative data shown for C10 founder) (**Fig. 2B**). Then, we expanded the C10 founder line, which showed higher expression of GFP, breeding it with wild-type mice. Next, we analyzed immunofluorescence against

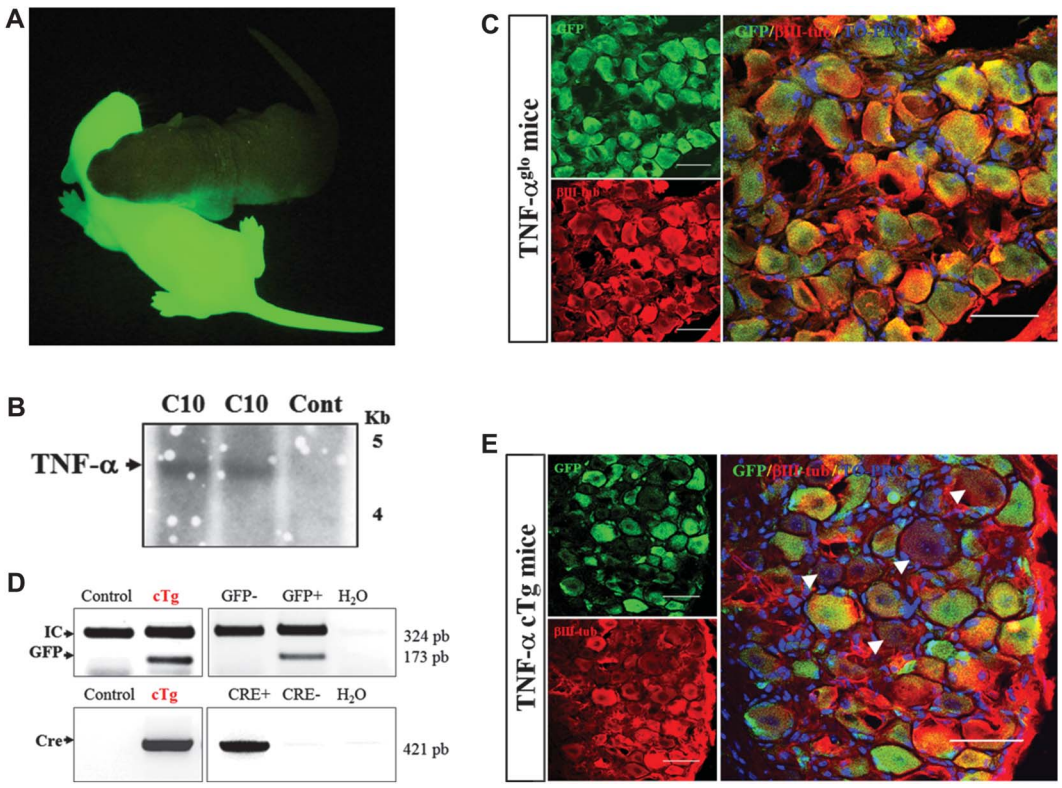

Figure 2. Generation of TNF-α cTg mice. (A) Representative image of 2 mice at P4 age, 1 TNF^{glo} mouse displays endogenous GFP fluorescence (green), whereas the control mouse does not, by using a Macro Imaging System. (B) Southern blot analysis of genomic DNA of TNF^{glo} founder (C10) and control (Cont) mice. A band near 4.6 kb was detected only in founder C10 mice. (C) Representative immunofluorescence of trigeminal ganglia from TNF-α mice at 3 months of age showing endogenous GFP fluorescence (green), βIII-tubulin antibody (neurons in red), and TO-PRO-3 (nuclei in blue). GFP expression is detected in all types of cells. (D) Genotyping of TNF-α cTg and control mice by using PCR for GFP and Cre. (E) Representative immunofluorescence of TG from TNF-α cTg mice at 3 months of age showing endogenous GFP fluorescence (green), βIII-tubulin antibody (neurons in red), and TO-PRO-3 (nuclei in blue). GFP expression is absent in some neurons where recombination ocurred driven by Nav1.8-cre (white arrow).

βIII-tubulin (red), a marker of neurons, and we were able to detect endogenous GFP fluorescence (green) in various cell types, including TG neurons from TNF^{glo} mice (**Fig. 2C**). Phenotypic analysis of transgenic mice demonstrated that they were born healthy and viable, without any signs of adverse effects of transgene integration.

3.2. Generation of TNF-α cTg mice

To generate a TNF-α cTg mice that overexpress TNF-α specifically in nociceptive neurons, we bred TNF^{glo} mice with a Nav1.8-Cre mice,[1] which express the Cre recombinase predominantly in nociceptive neurons in DRG and TG. Then, we genotyped pups by PCR by using GFP- and Cre-specific primers (**Fig. 2D**), and we found that mice were born with an expected Mendelian frequency (data not shown). By determination of immunofluorescence of TG from TNF-α cTg mice against βIII-tubulin (red) and endogenous GFP fluorescence (green), we found that some neurons (white arrow) were GFP negative, indicating removal of GFP by Cre recombination (**Fig. 2E**). TNF-α cTg mice were born healthy, and they were viable and fertile without any gross phenotype. We also evaluated the body weight of these mice starting at the weaning age until 3 months of age for both male and female mice and found no difference between TNF-α cTg and control mice (data not shown), suggesting no deleterious effects due to higher expression of TNF-α in nociceptive neurons.

3.3. TNF-α overexpression in nociceptive neurons of TNF-α cTg mice

We analyzed TNF-α mRNA and protein levels in TGs from TNF-α cTg and control mice at 3 months of age and found a significant increase in TNF-α mRNA (**Fig. 3A**) and protein (**Fig. 3B**) levels. Using immunofluorescence analysis, we found that TNF-α was expressed at detectable levels in trigeminal neurons of the control mice (**Fig. 3D**); however, TNF-α immunofluorescence stain was significantly elevated in TNF-α cTg mice at 3 months of age. White arrows indicated successful deletion of GFP by Cre recombinase resulting in an increased TNF-α expression (**Figs. 3C and E**). We then evaluated what type of cells expressed TNF-α in these mice. First, we detected peptidergic neurons by using CGRP immunostaining (yellow arrow) and nonpeptidergic neurons by using IB4 immunostaining of TG sections of TNF-α cTg mice (**Fig. 3F**). We found that TNF-α is highly expressed in all types of neurons, in particular, TNF-α colocalized with IB4-positive neurons and with also others neurons, possibly TRPA1- and CGRP-positive neurons in TG from TNF-α cTg mice (**Figs. 3F–H**). TNF-α plays a pivotal role during neuroimmune interaction through activation of NFκB signaling pathway.[24,55,64] Interestingly, we found that TNF-α overexpression resulted in a significantly increased staining of phospho-NFκB p65 in TGs of TNF-α cTg mice as compared with control mice of 3 months of age (**Fig. 3I**), which suggests that TNF-α triggers activation of NFκB signaling in vivo.

3.4. TNF-α overexpression results in inflammation in nociceptive tissues

Because of relevant functions of TNF-α in inflammatory pain,[7,24] we characterized the effects of TNF-α overexpression on inflammatory mediators. First, we evaluated the presence of macrophages and activation of satellite glial cells in TG from TNF-α cTg and control mice by using Iba1 (**Fig. 4A**) and GFAP (**Fig. 4B**) markers, respectively. We found that TG sections of TNF-α cTg mice had significantly increased staining of Iba1 in cells surrounding trigeminal neurons (**Fig. 4A**). Similarly, GFAP staining was detected in satellite glial cells surrounding trigeminal neurons, and it was significantly increased in TGs of TNF-α cTg mice (**Fig. 4B**), which suggests that TNF-α overexpression results in local inflammation in nociceptive tissues. Second, we analyzed the mRNA expression levels of inflammatory mediators known to be involved in inflammatory pain such as IL-12, IFN-γ, MCP-1, IL-6, IL-1β, IL-10, TGF-β1, LIF, OSM, and TNF-α, in TG and brain of TNF-α cTg and control mice (**Fig. 4C**). We used spleen sample as a positive control for all inflammatory mediators (data not shown). Our analysis revealed that besides TNF-α, IL-6 and MCP-1 mRNA levels were also significantly increased in TG of TNF-α cTg mice as compared with control mice (**Fig. 4C**, upper panel). In addition, we confirmed significant increase in IL-6 levels by immunofluorescence staining in TG of TNF-α cTg mice (**Fig. 4D**). Interestingly, there was no difference in mRNA levels of all cytokines assayed in brains of these mice (**Fig. 4C**, bottom panel). We also evaluated whether TNF-α overexpression in nociceptive neurons in DRG and TG could increase levels of TNF-α and other cytokines in blood. There was no difference in TNF-α levels between TNF-α cTg (5.5 ± 1.0 pg/mL) and control (5.0 ± 2.2 pg/mL) mice at 1 month of age. However, at 3 months of age, the TNF-α levels increased in TNF-α cTg (14.3 ± 1.9 pg/mL) mice as compared with controls (6.3 ± 1.7 pg/mL) (**Fig. 5A**) although such increased level was within the reported physiological range for TNF-α levels.[67] Nonetheless, we decided to evaluate whether this TNF-α increase deregulates the immune homeostasis. We performed a Cytometric Bead Array (CBA) to analyze the expression profile of 6 important inflammatory mediators (IL-12, TNF-α, IFN-γ, MCP-1, IL-6, and IL-10) in blood, which are known to change at the initial onset of septic shock.[62] We found there was no difference in the levels of these inflammatory mediators in TNF-α cTg mice as compared with littermate controls (**Fig. 5B**). We could not detect IL-10 levels in both genotype mice, probably due to its low concentration. Taken together, these results demonstrate that TNF-α overexpression in the nociceptive tissues did not alter the immune homeostasis in TNF-α cTg mice.

3.5. TNF-α overexpression increased ERK1/2 and Egr1 signaling in nociceptive tissues

TNF-α has been implicated in inducing pain through activation of several MAPK signaling pathways in nociceptive tissues.[24,45,69] We and others have shown that activation of canonical pathways for Cdk5 kinase activity is related to ERK1/2 activation.[14,49,51] Moreover, we also reported TNF-α-mediated ERK1/2 activation in PC12 cells with subsequent increase in p35 protein and Cdk5 activity.[49,54] These findings prompted us to determine whether TNF-α overexpression could activate ERK1/2 pathway in TG from TNF-α cTg mice. Using immunofluorescence analysis, we found a significant increase in phospho-ERK1/2 staining (yellow arrow, **Figs. 6A and B**) in TG from TNF-α cTg mice at 3 and 6 months of age as compared with control mice. Interestingly, the activation of ERK1/2 pathway resulted in increased levels of transcription factor Egr1. We further confirmed increased Egr1 levels by immunofluorescence. We found that TNF-α overexpression significantly increased neuronal immunodetection of Egr1 and its nuclear localization (yellow arrows, **Fig. 6C**) at 3 and 6 months of age in TG from TNF-α cTg mice as compared with controls (**Figs. 6C and D**).

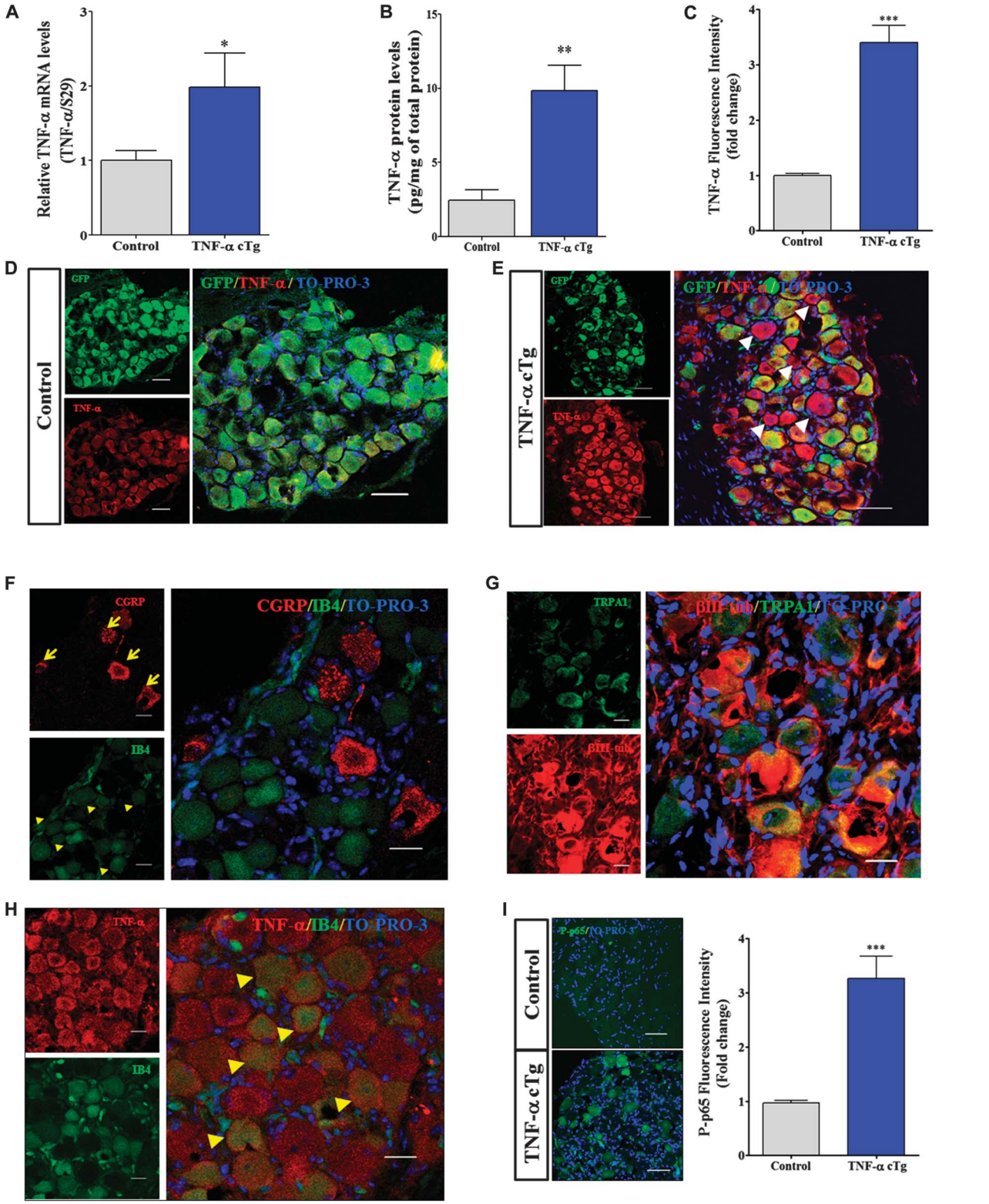

Figure 3. TNF-α overexpression in TG from TNF-α cTg mice. (A) Relative TNF-α mRNA levels in trigeminal ganglia (TGs) from TNF-α cTg and control mice at 3 months of age (n = 3-7 mice). S29 mRNA was used as a control. (B) TNF-α protein expression in TG from TNF-α cTg and control mice at 3 months of age measured by enzyme linked immunosorbent assay (n = 4-7 mice). (C) Quantification of TNF-α immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 months of age (n = 2 mice, between 21-42 neurons). Representative immunofluorescence of TNF-α (red), endogenous GFP fluorescence (green), and TO-PRO-3 (blue) of TG from control (D) and TNF-α cTg (E) mice at 3 months of age. White arrow shows Cre recombination and overexpression of TNF-α immunodetection. Representative immunofluorescence of: (F) CGRP (red) and IB4 (green); (G) TRPA1 (red) and βIII-tubulin; and (H) TNF-α (red) and IB4 (green) of TG from TNF-α cTg mice at 3 months of age (bar = 50 μm). (I) Representative immunofluorescence of phospho-p65 (P-p65, green) of TG from TNF-α cTg and control mice at 3 months of age and the quantification of P-p65 immunodetection (n = 2 mice, between 22-30 neurons). The bar graphs represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ as compared with control mice.

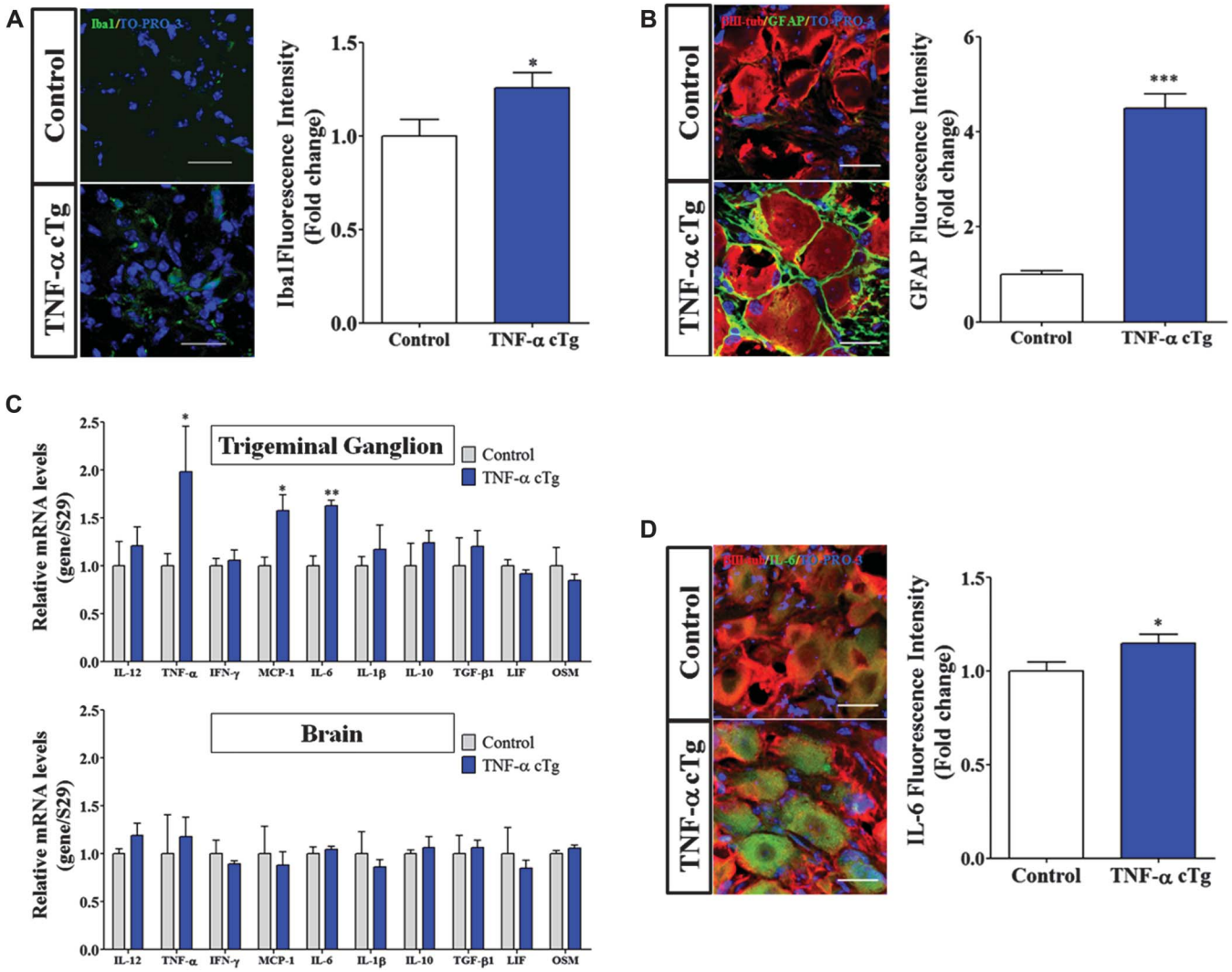

Figure 4. TNF-α overexpression caused inflammation in trigeminal ganglia (TGs) from TNF-α cTg mice. (A) Representative immunofluorescence of Iba1 (green), a marker of activated microglia/macrophages and nuclei (TO-PRO-3, blue) in TG from TNF-α cTg and control mice at 3 months of age (bar = 50 μm) and quantification of Iba1 immunodetection (n = 2 mice, between 17-23 neurons). (B) Representative immunofluorescence of GFAP (green), a marker of activated satellite glial cells and nuclei (TO-PRO-3, blue) in TG from TNF-α cTg and control mice at 3 months of age (bar = 50 μm) and quantification of GFAP immunodetection (n = 2 mice, between 21-22 neurons). (C) Quantification of mRNA levels of inflammatory mediators in TG and brain from TNF-α cTg and control mice at 3 months of age. We analyzed inflammatory mediators such as IL-12, TNF-α, IFN-γ, MCP-1, IL-6, IL-1β, IL-10, TGF-β1, LIF, and OSM. S29 mRNA was used as a housekeeping gene. (D) Representative immunofluorescence of IL-6 (green), βIII-tubulin (red, neurons), and nuclei (TO-PRO-3, blue) of TG from TNF-α cTg and control mice at 3 months of age and quantification of IL-6 immunodetection (n = 2 mice, between 26-27 neurons). The bar graphs represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ as compared with control mice.

3.6. TNF-α overexpression increased p35 and Cdk5 levels and Cdk5 kinase activity

Because we had earlier found that Egr1 regulates p35 expression,[14,49,51] we analyzed p35 and Cdk5 levels in TG from TNF-α cTg mice by immunofluorescence and Western blot. We found a significant increase in Cdk5 (**Figs. 7A and B**) and p35 immunostaining (**Figs. 7C and D**) in TG from TNF-α cTg as compared with controls at 3 and 6 months of age by using immunofluorescence analysis. We also performed Western blot analysis for Cdk5 and p35 protein levels and found that Cdk5 protein levels were not changed in both genotypes (**Fig. 7E**). However, p35 protein level was significantly increased in TG from TNF-α cTg as compared with control mice at 3 months of age (**Fig. 7F**). Most importantly, we found that Cdk5 kinase activity was significantly increased in TG from TNF-α cTg as compared with control mice at 3 months of age (**Fig. 7G**), which indicates that TNF-α overexpression increases Cdk5 kinase activity in vivo, possibly by an increase in p35 levels.

3.7. TNF-α overexpression increased Cdk5-mediated phosphorylation of TRPV1 at T407

We previously discovered that Cdk5 phosphorylates TRPV1 at T407, which is believed to increase Ca^{2+} influx in cultured DRG neurons and odontoblast-like cells.[34,51,53] Therefore, we assessed whether increased Cdk5 activity in TNF-α cTg mice elevates phosphorylation of TRPV1 at T407 in TG and in the cultured TG neurons from TNF-α cTg mice. First, we found no change in TRPV1 expression by Western blot (data not shown), and also total TRPV1 staining in TGs was not altered in TNF-α cTg mice (**Figs. 8A and B**). However, we found significant increase in phospho-T407-TRPV1 in TG of TNF-α cTg mice as compared

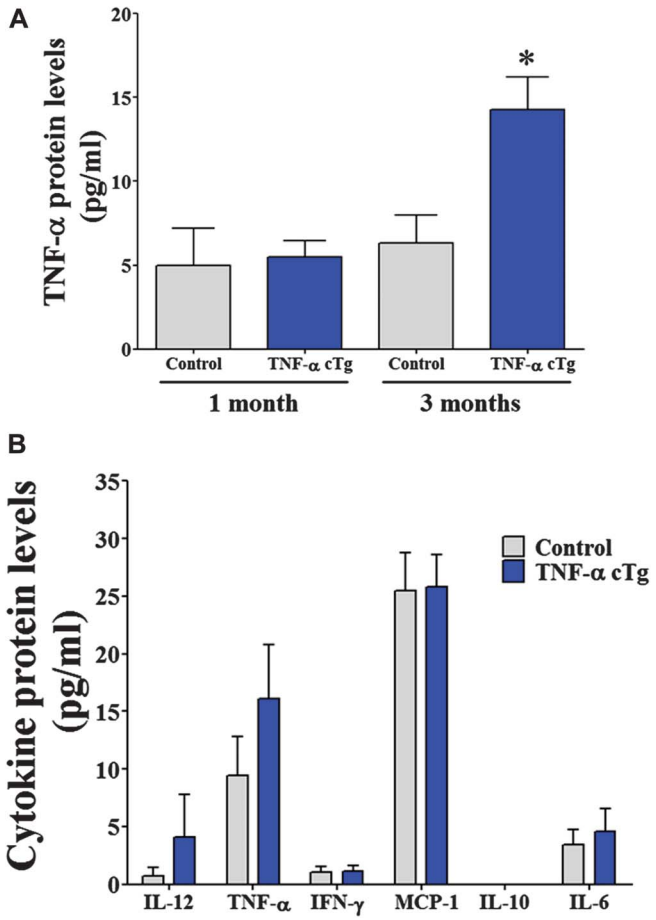

Figure 5. TNF-α overexpression in TNF-α cTg mice does not affect systemic immune status. (A) Quantification of TNF-α protein expression in the serum from TNF-α cTg and control mice at 1 and 3 months of age measured by TNF-α enzyme linked immunosorbent assay (n = 5-6 mice). (B) Quantification of the expression of 6 inflammatory mediators (IL-12, TNF-α, IFN-γ, MCP-1, IL-6, and IL-10) in the serum from TNF-α cTg and control mice at 3 months of age by using the Cytometric Bead Array (CBA). IL-10 was not detected (n = 4-6 mice). The bar graphs represent mean ± SEM. *$P < 0.05$ as compared with control mice.

with control mice at 3 months of age (**Figs. 8C and D**). Additionally, we evaluated whether increased Cdk5 activity affects phosphorylation and function of TRPV1 in cultured TG neurons from TNF-α cTg mice. First, we cultured TG of 3-month-old neurons from TNF-α cTg and control mice for 18 hours, and then we performed immunofluorescence for phospho-T407-TRPV1 (blue) and βIII-tubulin (red) in these cultures (**Fig. 8E**). Interestingly, we found a significant increase in phospho-T407-TRPV1 staining in TG neurons, that were GFP negative (see white arrows in **Fig. 8F**), from TNF-α cTg mice as compared with control mice (**Fig. 8F**), which suggests that Cdk5 activity can regulate phosphorylation of TRPV1 in TG tissue and cultured TG neurons.

3.8. Cdk5/p35 complex increased sensitivity to capsaicin of TRPV1-transfected HEK-293 cells

To evaluate the effect of Cdk5 activity over TRPV1 function, we performed Ca^{2+} imaging experiments in the heterologous system of HEK-293 cells cotransfected with TRPV1-GFP and p35 or empty vector (control) by using TRPV1 agonist capsaicin. We showed that the expression of p35 causes an increase in the sensitivity to capsaicin of TRPV1-expressing cells, revealed by the higher response to this vanilloid at low concentrations (2 nM) compared with control cells (**Figs. 9A and B**). Moreover, 49% (53/108) of the cells responding to 200 nM capsaicin also respond to 2 nM capsaicin in p35(+) cells, whereas only 32% (30/94) of control cells show sensitivity to this subsaturating concentration of the vanilloid ($P < 0.05$, F test) (**Figs. 9A and B**). However, the responses to 20 nM and 200 nM are similar, which indicates that 20 nM of capsaicin is a saturating stimulus in this system. These results suggest that the activation of Cdk5 by p35 enhances the response of TRPV1 to capsaicin, probably by phosphorylation of the channel.[34]

3.9. TNF-α overexpression increased TRPV1-dependent Ca^{2+} influx in cultured trigeminal neurons

We performed Ca^{2+} influx analyses on acutely cultured TG neurons (4 hours after plating) from TNF-α cTg and control mice, treated with capsaicin and finally with KCl as a control of viability (**Fig. 9C**). We first evaluated the response of TG neurons to a saturating concentration of capsaicin (200 nM), and we found that the percentage of cultured TG neurons responding to capsaicin was similar in control (46.2%) and TNF-α cTg mice (36.5%) (data not shown). However, when we stimulated cultured TG neurons with a subsaturating concentration of capsaicin (20 nM), we found that the percentage of capsaicin-responsive neurons was significantly higher in trigeminal neurons from TNF-α cTg (35.1%) compared with control mice (10.5%) (**Fig. 9D**). Then, we performed the same experiment in the presence or absence of the Cdk5 inhibitor roscovitine (10 μM) that was present during 4 hours before Ca^{2+} influx analyses. We did not find difference in the percentage of capsaicin-responding neurons in control TG neurons treated with roscovitine (17.6%) as compared with untreated control TG neurons (10.5%). However, pretreatment of TG cultures from TNF-α cTg with roscovitine (10 μM) prevented the increase in the percentage of capsaicin-responding neurons from the untreated group (from 35.1% to 6.7%), which suggests that Cdk5 regulates the TRPV1-dependent Ca^{2+} influx by TRPV1 phosphorylation (**Fig. 9D**). Finally, we did not find change in the amplitude of capsaicin-evoked $[Ca^{2+}]_i$ elevations at 20 nM in both genotypes (data not shown).

3.10. TNF-α overexpression in TG increased orofacial thermal pain sensation

We performed operant behavioral tests on TNF-α cTg and control mice of 6 to 8 months old by using the OPAD, previously used to measure orofacial thermal pain in rodents.[2] After 15 ± 1 hours of water and food withdrawal, we measured reward licking events (number of licks) at 2 temperatures: 33°C (nonaversive) during 3 minutes followed by 45°C (aversive) during 3 minutes in control and TNF-α cTg mice. We found that control and TNF-α cTg mice perform higher number of licks at 33°C, and there is no difference between genotypes. However, there is a significant reduction in the number of licks at 45°C in both control and TNF-α cTg mice, being smaller on TNF-α cTg mice (data not shown). Most importantly, we evaluated reward licking events (**Fig. 10A**), the lick-to-face contact ratio (**Fig. 10B**), and total water intake (**Fig. 10C**) between control and TNF-α cTg mice during a 20-minute test at 45°C (aversive temperature). We found a significant reduction of all these parameters on TNF-α cTg mice as

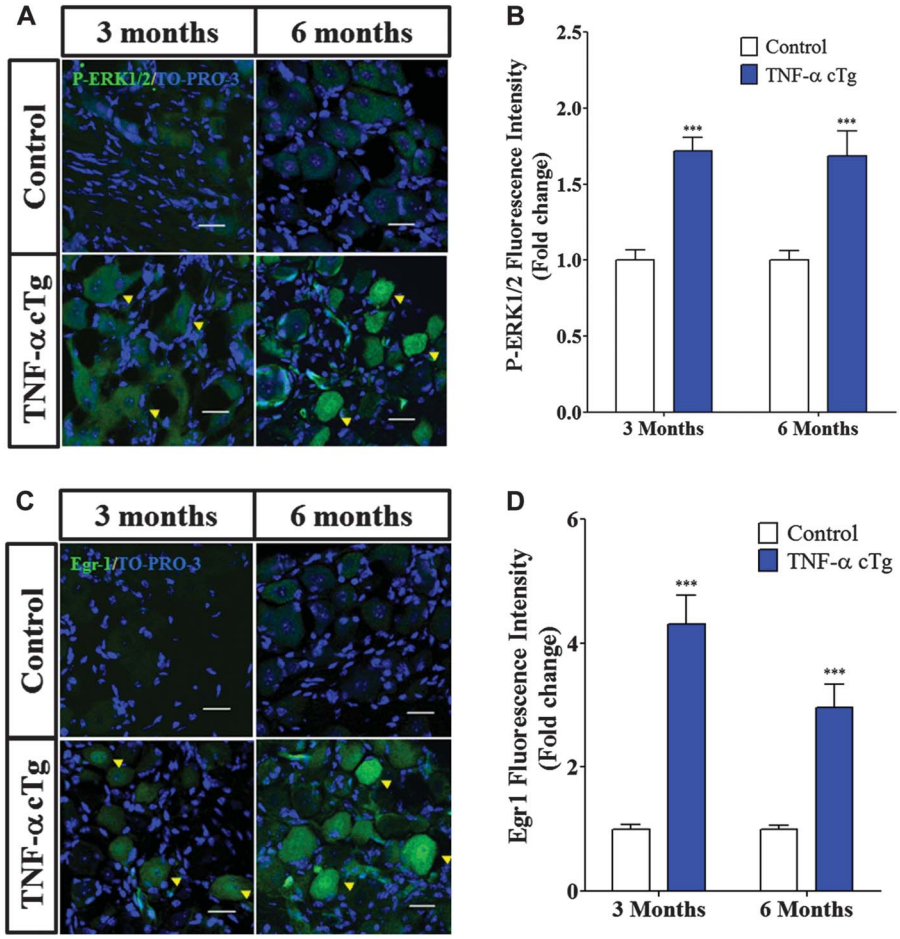

Figure 6. TNF-α overexpression activates ERK1/2 pathway and increases Egr1 expression in trigeminal ganglia (TGs) from TNF-α cTg mice. (A) Representative immunofluorescence of phospho-ERK1/2 (green) and nuclei (TO-PRO-3, blue) in TG from TNF-α cTg and control mice at 3 and 6 months of age. Yellow arrows show increased staining in TG from TNF-α cTg mice. (B) Quantification of phospho-ERK1/2 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 and 6 months of age (n = 2 mice, between 21-23 neurons). (C) Representative immunofluorescence of Egr1 (green) and nuclei (TO-PRO-3, blue) in TG from TNF-α cTg and control mice at 3 and 6 months of age. Yellow arrows show nuclear translocation of Egr1 in TG from TNF-α cTg mice. (D) Quantification of Egr1 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 and 6 months of age (n = 2 mice, between 20-21 neurons) (bar = 50 μm). The bar graphs represent mean ± SEM. ***$P < 0.001$ as compared with control mice (2-way analysis of variance, with Bonferroni posttest).

compared with control mice, which indicates the important role of TNF-α in orofacial thermal sensitivity.

4. Discussion

We and others discovered that Cdk5 plays an important role during inflammation-induced pain signaling.[36,38,48,66] Moreover, we identified the relevance of cytokines to Cdk5 activation by showing that TNF-α and TGF-β1 increase Cdk5 activity through ERK1/2 signaling, which in turn increases Egr1 and p35 expression in PC12 cells, odontoblast-like cells, and DRG cultured neurons.[49,51,53,54] It has also been shown that recombinant TNF-α can activate ERK1/2 signaling in cultured TG neurons from rats, in which it increases CGRP expression.[23] Moreover, tissue injury or inflammation increases Egr1 expression in nociceptive neurons, and this correlates with persistent pain and hyperalgesia.[9,29] In addition, pain signaling at the level of the spinal cord seems to be dependent on Cdk5 activity.[10,25] Interestingly, knocking down Cdk5 in the prelimbic cortex, which is involved in the central processing of pain sensation, during paw inflammation in rats decreased pain-related behavior.[56] This accumulating evidence of involvement of the Cdk5/p35 system in pain at the peripheral and central levels makes it an interesting potential therapeutic target. Our current findings support this notion by demonstrating linkage of TNF-α overexpression to increased Cdk5/p35 activity associated with orofacial thermal hyperalgesia. These data support reports showing reduced pain after roscovitine treatment in several rodent models of neuropathic and inflammatory pain.[25,27,65,71]

The relevance of TNF-α/Cdk5 axis in pain can be explained in part by the Cdk5-dependent phosphorylation of several substrates involved in pain signaling such as KIF13B,[61] delta opioid receptor,[60] P2X3 purinergic receptor,[32] NMDA receptor subunit (NR2A and NR2B), and mGluR5,[27] promoting Cdk5 as a potential target for developing new analgesics.[48] One of the most relevant of these substrates is TRPV1 which is preferentially phosphorylated at T407 by Cdk5, and this phosphorylation is implicated in activation of TRPV1, which is involved in regulation of Ca^{2+} influx in cultured DRG sensory neurons and odontoblast-like cells,[34,51,53] and also increasing intracellular trafficking of TRPV1 channels from vesicles to the plasma membrane, thereby promoting thermal hyperalgesia.[26,61]

In this study, we set out to determine whether TNF-α modulates Cdk5 activity in vivo. Current mouse models that

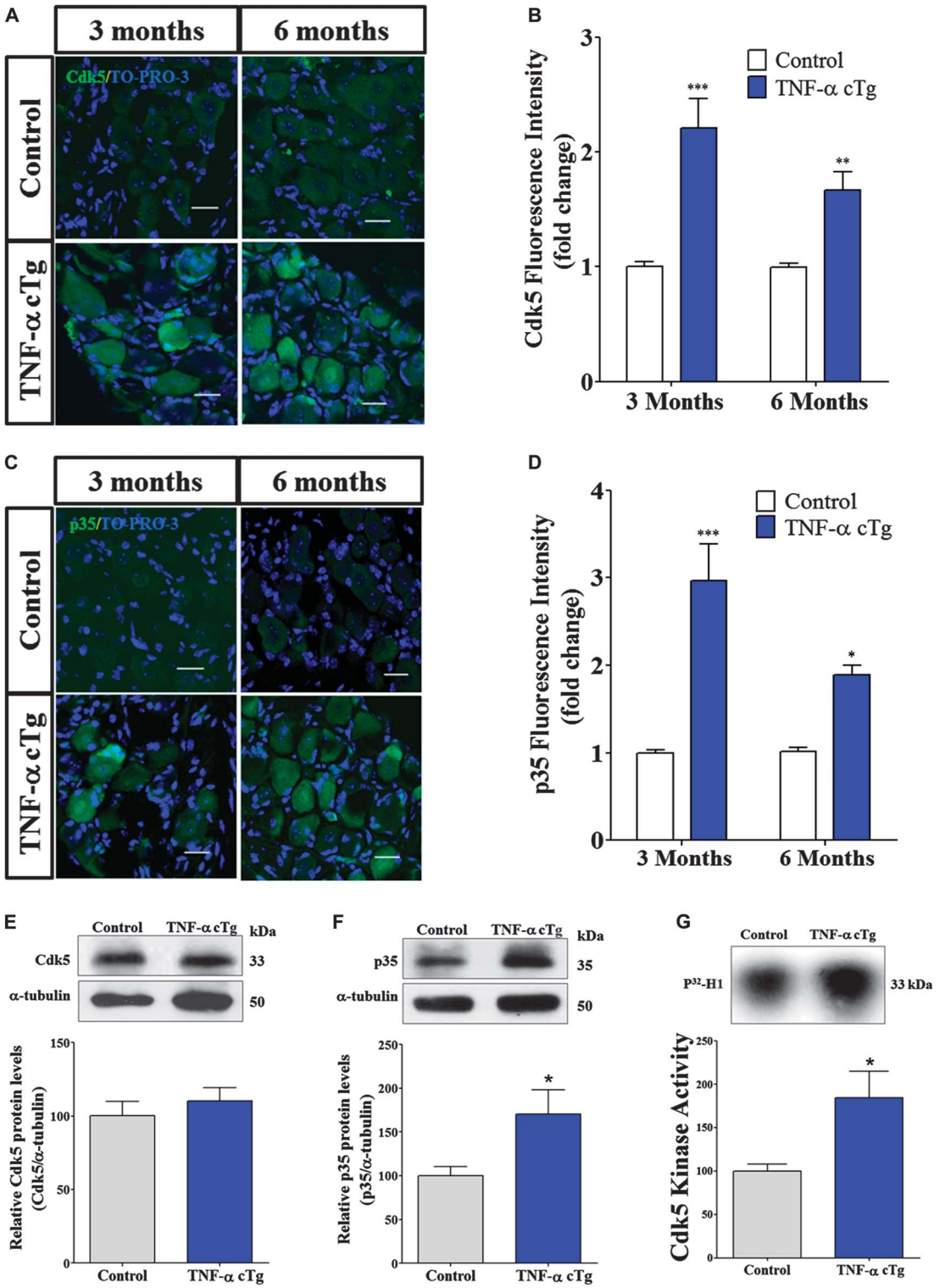

Figure 7. TNF-α overexpression increases Cdk5 and p35 expression and Cdk5 kinase activity in trigeminal ganglia (TGs) from TNF-α cTg mice. (A) Representative immunofluorescence of Cdk5 (green) in TG from TNF-α cTg and control mice at 3 and 6 months of age (bar = 50 μm). (B) Quantification of Cdk5 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 and 6 months of age (n = 2 mice, between 21-26 neurons). (C) Representative immunofluorescence of p35 (green) in TG from TNF-α cTg and control mice at 3 and 6 months of age (bar = 50 μm). (D) Quantification of p35 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 and 6 months of age (n = 2 mice, between 34 and 42 neurons). The bar graphs represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ as compared with control littermate mice (2-way ANOVA 2 tail, with Bonferroni posttest). (E) Upper panel: Representative Western blots for Cdk5 and α-tubulin protein expression of TG from TNF-α cTg and control mice at 3 months of age. Bottom panel: Western blot quantification for Cdk5 (n = 3-4 mice). (F) Upper panel: Representative Western blots for p35 and α-tubulin protein expression of TG from TNF-α cTg and control mice at 3 months of age. Bottom panel: Western blot quantification of p35 (n = 9 mice). (G) Upper panel: Representative in vitro Cdk5 kinase activity of TG immunoprecipitates with Cdk5 antibody from TNF-α cTg and control mice at 3 months of age. Bottom panel: Quantification of Cdk5 activity of TG from TNF-α cTg as compared with control mice at 3 months of age (n = 3-4 mice). The bar graphs represent mean ± SEM. $^{*}P < 0.05$, as compared with control littermate mice (Student *t* test).

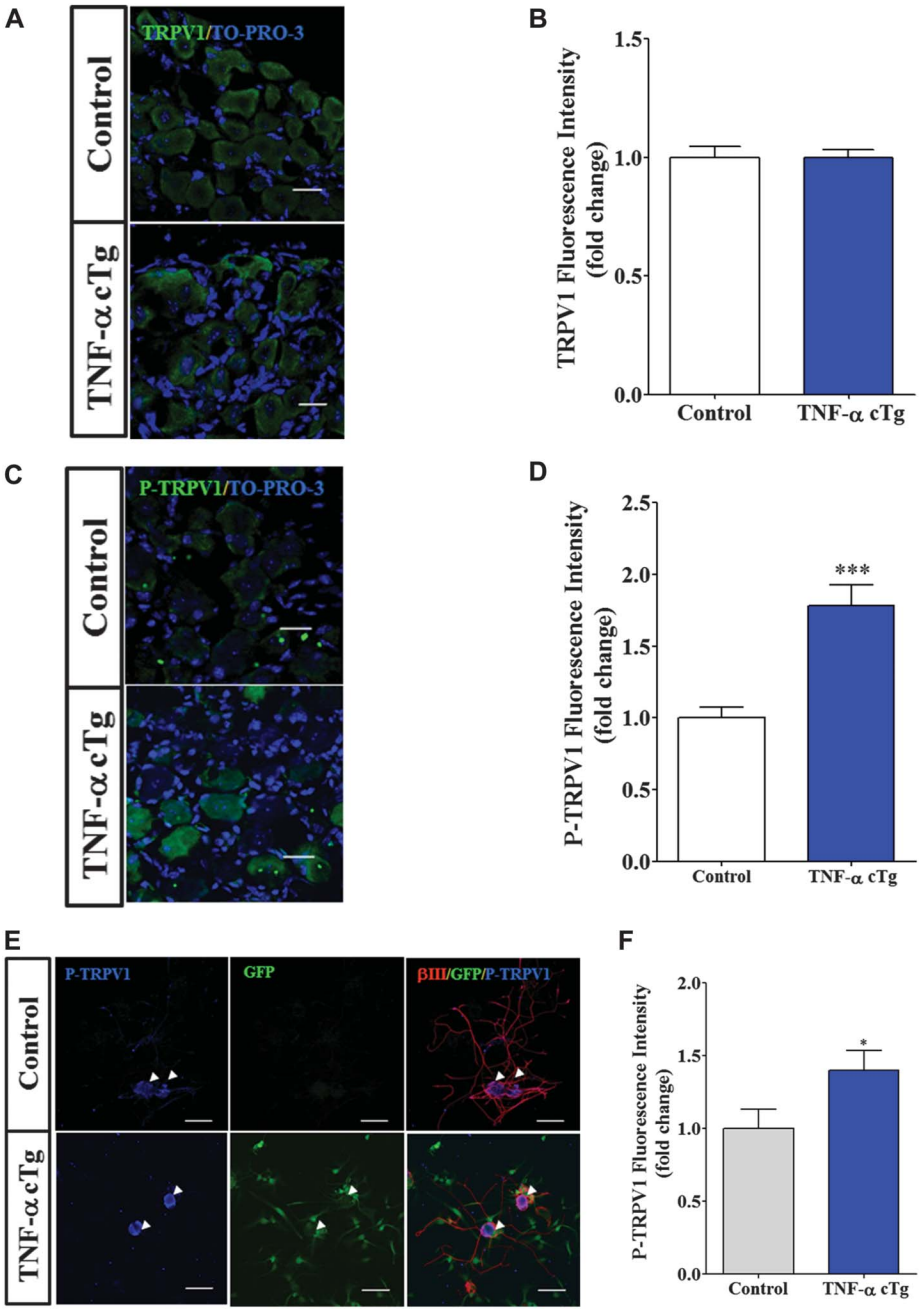

Figure 8. TNF-α overexpression increases Cdk5-mediated TRPV1 phosphorylation at T407 in trigeminal ganglia (TGs) and in cultured TG primary neurons from TNF-α cTg mice. (A) Representative immunofluorescence of TRPV1 (green) in TG from TNF-α cTg and control mice at 3 months of age (bar = 50 μm). (B) Quantification of TRPV1 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 months of age (n = 2 mice, between 22-33 neurons). (C) Representative immunofluorescence of phospho-T407-TRPV1 (green) in TG from TNF-α cTg and control mice at 3 months of age (bar = 50 μm). (D) Quantification of phospho-T407-TRPV1 immunodetection in TG from TNF-α cTg mice as fold change compared with control mice at 3 months of age (n = 2 mice, between 36-43 neurons). (E) Representative immunofluorescence of phospho-T407-TRPV1 (blue), βIII-tubulin (red), and endogen GFP fluorescence (green) in cultured TG neurons from TNF-α cTg and control mice during 18 hours. White arrow shows increase of phospho-T407-TRPV1 immunodetection in TG neurons from TNF-α cTg mice (bar = 50 μm). (F) Quantification of phospho-T407-TRPV1 immunodetection in cultured TG neurons from TNF-α cTg as compared with control neurons (n = 1 mouse, between 28-30 neurons). The bar graphs represent mean ± SEM. *$P < 0.05$ and ***$P < 0.001$ as compared with control mice (Student *t* test).

have been developed to understand the role of TNF-α in inflammatory pain used traditional approaches such as administering CFA, carrageenan, or formalin into the paws, or TNF-α administration at intrathecal, subcutaneous, or cranial meninges sites.[11,31,63,68,70] However, these models proved to be transient with a considerable variation over a period. To overcome this problem, we developed a different strategy to genetically engineer a mouse model for studies that involve TNF-α induced pain. We designed a new conditional transgenic mouse that overexpresses TNF-α specifically in nociceptive neurons using the Cre-loxP system. Previously, others transgenic mouse models has been created using the same pCLE vector, such as huntingtin-interacting protein-1 (HIP-1),[3] TGF-β1^{glo} mice,[13] and recently TNF-α^{glo} mice in

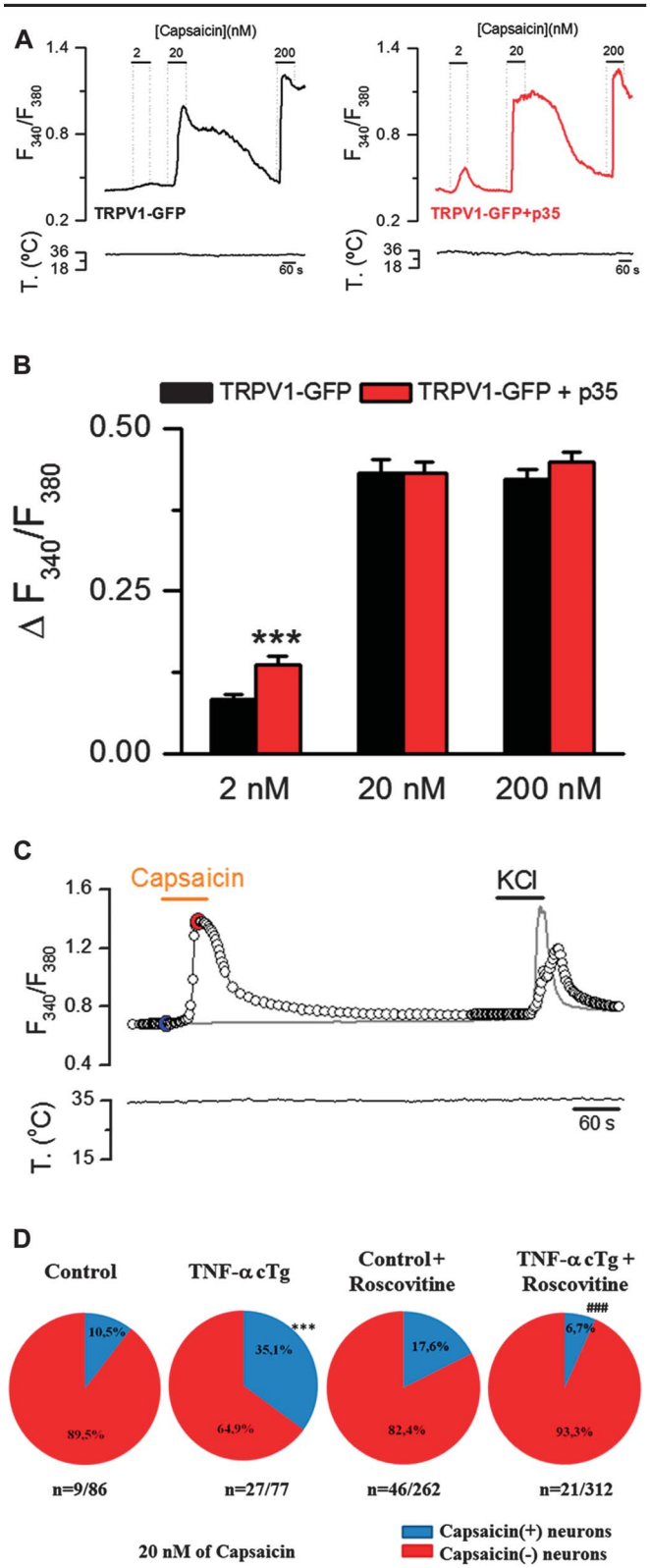

Figure 9. Cdk5/p35 potentiates TRPV1 sensitivity to capsaicin in HEK-293 cells and in cultured trigeminal ganglion (TG) neurons from TNF-α cTg mice. (A) Representative intracellular calcium imaging traces showing the response to 2, 20, and 200 nM capsaicin in HEK-293 cells expressing TRPV1-GFP (black, left panel) and TRPV1-GFP coexpressed with p35 (red, right panel). (B) Summary histogram of the results obtained for the experimental protocol in A. Statistical significance was assessed with a 2-way ANOVA test. Δ ratio of control condition (n = 94) is different (***$P < 0.001$) with respect to p35 condition (n = 108). Recordings from both conditions were always interlaced and performed on the same days. (C) Radiometric $[Ca^{2+}]_i$ response of a capsaicin-sensitive (TRPV1[+]) (black) and a capsaicin-insensitive (gray) TG neuron to 20 nM capsaicin. Basal $[Ca^{2+}]_i$ level and maximal response to capsaicin correspond with the points marked in blue and red, respectively. A high (30 mM) KCl pulse was included at the end of the protocol to evaluate the depolarization-induced response of the entire population of TG neurons in the field. (D) Pie chart showing the number of TG neurons responding to capsaicin (20 nM) in the presence or absence of roscovitine (10 μM), added 4 hours before Ca^{2+} imaging experiments (n = 3-6 mice for each genotype, between 63-312 neurons analyzed). ***$P < 0.005$ as compared with control littermate mice (Fisher test). ###$P < 0.005$ as compared with TNF-α cTg mice (Fisher test).

odontoblast and osteoclast cells.[12] To conditionally overexpress TNF-α in nociceptive neurons, we bred TNF-α^{glo} mice with Nav1.8-Cre mice, which are known to express Cre in all small-diameter neurons and a small percentage of large-diameter nociceptive neurons in the DRG and TG.[1]

Our strategy to generate TNF-α cTg mice was validated by the increased expression of TNF-α and its activation of the NFκB signaling pathway that we observed in the TG of these mice and in the TNF-α/DMP1-Cre mice developed in parallel to our study.[12] We also detected increased levels of IL-6 and MCP-1, activation of satellite glial cells (GFAP staining), and the presence of macrophages (Iba1 staining) in the TG from TNF-α cTg mice suggesting neuroinflammation. Interestingly, increased levels of TNF-α, IL-6, MCP-1, and Iba1 were observed in the TG from a migraine mouse model, which suggests that TNF-α might modulate sensory neurons and resident glial cells, underlying the process of neuronal sensitization.[11] Nevertheless, in this migraine mouse model, IL-10 and IL-1β mRNA were also upregulated, indicating that different mechanisms could be operating in these different genetic approaches. TNF-α can increase IL-6 expression through NFκB activation in osteoclast-like cells[20] and also it can increase MCP-1 levels in endothelial cells.[21] Furthermore, TNFR1 antisense oligonucleotides can decrease IL-6 regulation through NFκB signaling in a rat neuropathic pain model.[22] However, we found that TNF-α overexpression in nociceptive neurons does not alter systemic immune homeostasis[62] as determined by TNF-α ELISA and CBA assays from the serum of TNF-α cTg and control mice. Taken together, our findings of trigeminal sensitization and a neuroinflammation in TG without a systemic response in TNF-α cTg mice make them a good model for studying diverse painful conditions such as inflammatory pain or neuropathic pain induced by nerve damage in a sensitized context.

One possible mechanism that explains the sensitization observed when TNF-α pathway is activated could involve the modulation of TRPV1 channels. In our experiments using heterologous expression of TRPV1 in HEK293 cells, calcium influx mainly depends on channel activation by capsaicin. In this system, coexpression with p35 resulted in an increase in the responses of the cells to a subsaturating concentration of this vanilloid, compared with control condition. In the same line, our results in trigeminal neurons from wild-type and TNF-α cTg mice shows that the percentage of capsaicin-sensitive neurons is larger in transgenic mice than in control animals at a subsaturating concentration of this TRPV1 activator. Although a difference in the magnitude of these responses could be expected, their amplitude in control and transgenic mice were virtually identical at this concentration of capsaicin. However, we have to consider that calcium imaging is only an indirect manner to study the function of a particular calcium-permeable pathway in these neurons. Probably, once the voltage threshold for firing in TRPV1-expressing neurons is

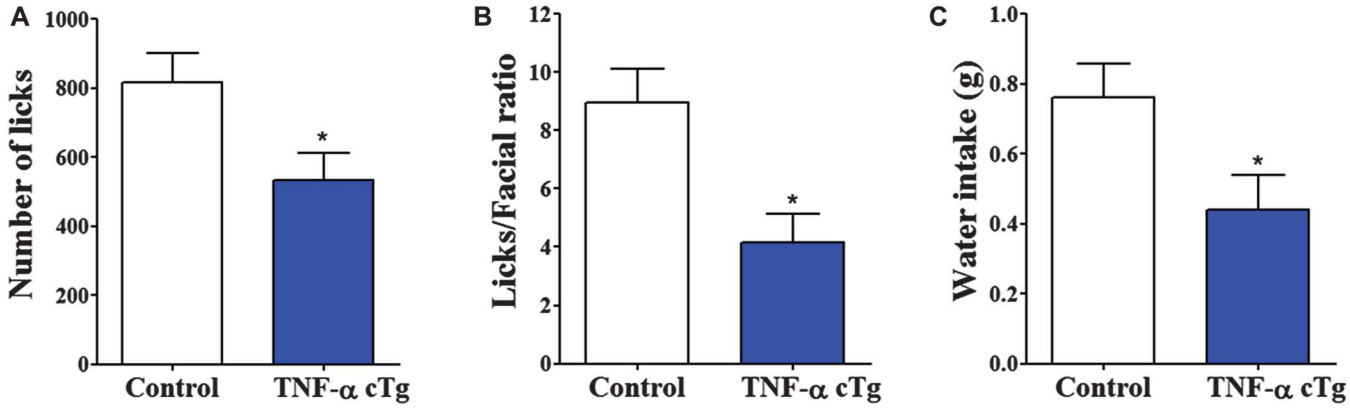

Figure 10. TNF-α overexpression increased orofacial thermal pain sensation in TNF-α cTg mice. (A) Quantification of licking events at 45°C (aversive) during 10 minutes. (B) Licking/face ratio at 45°C (aversive) during 10 minutes, and (C) water intake (in grams) between control and TNF-α cTg mice during 10-minute test at 45°C. The bar graphs represent mean ± SEM. (n = 4-5 mice) *$P < 0.05$ as compared with control mice (Student *t* test).

reached, calcium entry due to the activation of voltage-gated Ca^{2+} channels during action potentials is similar in both conditions, yielding this result. Thus, although calcium imaging is a useful tool to study TRPV1-dependent responses in these systems, further studies will be necessary in the future to address TRPV1 sensitivity to capsaicin (and heat) in a more direct and quantitative manner, using the patch clamp technique, to understand the bases of the modulation of this channel by TNF-α through Cdk5/p35 pathway in native membranes. Despite the relatively modest change in the capsaicin response in cultured TG neurons, TNF-α cTg mice displayed orofacial thermal hyperalgesia at basal levels indicating trigeminal sensitization by chronic TNF-α overexpression. This unexpected mild response to capsaicin in primary cultures could be explained by different inflammatory contexts between neurons at the TG and isolated cultured neurons. The sensitization at the TG could be due to autocrine or paracrine TNF-α signaling in nociceptive neurons, but it could also be mediated by other cell populations at the TG. Importantly, macrophages can become activated by TNF-α triggering the secretion of proinflammatory mediators such as other cytokines, chemokines, and TNF-α itself during painful conditions.[24,30,59] Satellite glial cells can also respond to TNF-α stimulation by secreting inflammatory factors such as TNF-α and IL-1β.[47] Another cell population participating in TG environment is vascular endothelial cells. These cells respond to MCP-1 increasing vasodilatation and vascular permeability,[44,58] facilitating leukocyte infiltration. Thus, a vicious cycle could be established by making trigeminal neurons sensitized also by a cell nonautonomous effect.

Time factor is an important point to consider when modeling inflammatory and neuropathic painful conditions. Contrary to most mouse models currently used, our TNF-α cTg mice not only overexpress TNF-α chronically but also during neurodevelopment. This early and sustained overexpression can induce compensatory and/or desensitization mechanisms that can explain the unexpected nonaggressive phenotype for TNF-α overexpression. This long-lasting TNF-α expression can be a desirable feature for studying chronic pain states in which several inflammatory components are deregulated (reviewed in Ref. 19). Because the Nav1.8 promoter is also expressed in sensory neurons of the DRG, we expect that our findings related to trigeminal-mediated pain can be applied to the DRG and dorsal horn of the spinal cord. Therefore, our TNF-α cTg mouse model will be a valuable tool to study molecular mechanisms of inflammatory pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

P. Rozas and P. Lazcano contributed equally to this study.

This work was supported by FONDECYT 11110136 and 1151043, and PAI-79100009 (to E.U.), and FONDECYT 1140325 and ACT-1114 (to C.G.-B.); FONDECYT 1131064 (to R.M.) and ACT-1113 (to R.M. and M.P.); FONDECYT 11130144 (to M.P.); and the Division of Intramural Research, National Institute of Dental and Craniofacial Research, National Institutes of Health.

Acknowledgements

The authors thank Dr Rohini Kuner for Nav1.8-Cre mice, Dr Andrzej Dlugosz for the pCLE-GFP plasmid, Bradford Hall for advice in TNF-α cloning strategy, Dr Maria Rosa Bono for CBA assays, Dr Michaela Prochazkova and Jason Keller for helpful discussion, and Monserrat Barrios for technical support.

Article history:
Received 8 July 2015
Received in revised form 8 February 2016
Accepted 9 February 2016
Available online 17 February 2016

References

[1] Agarwal N, Offermanns S, Kuner R. Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 2004;38:122–9.
[2] Anderson EM, Mills R, Nolan TA, Jenkins AC, Mustafa G, Lloyd C, Caudle RM, Neubert JK. Use of the Operant Orofacial Pain Assessment Device (OPAD) to measure changes in nociceptive behavior. J Vis Exp 2013;76: e50336.
[3] Bradley SV, Hyun TS, Oravecz-Wilson KI, Li L, Waldorff EI, Ermilov AN, Goldstein SA, Zhang CX, Drubin DG, Varela K, Parlow A, Dlugosz AA, Ross TS. Degenerative phenotypes caused by the combined deficiency of murine HIP1 and HIP1r are rescued by human HIP1. Hum Mol Genet 2007;16:1279–92.
[4] Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816–24.
[5] Contreras-Vallejos E, Utreras E, Borquez DA, Prochazkova M, Terse A, Jaffe H, Toledo A, Arruti C, Pant HC, Kulkarni AB, Gonzalez-Billault C.

Searching for novel Cdk5 substrates in brain by comparative phosphoproteomics of wild type and Cdk5-/- mice. PloS One 2014;9: e90363.
[6] Cho A, Haruyama N, Kulkarni AB. Generation of transgenic mice. Curr Protoc Cell Biol 2009. Chapter 19.11.1–19.
[7] Chu WM. Tumor necrosis factor. Cancer Lett 2013;328:222–5.
[8] Dhavan R, Tsai LH. A decade of CDK5. Nat Rev Mol Cell Biol 2001;2: 749–59.
[9] Dolan S, Hastie P, Crossan C, Nolan AM. Co-induction of cyclooxygenase-2 [correction of cyclooxyenase-2] and early growth response gene (Egr-1) in spinal cord in a clinical model of persistent inflammation and hyperalgesia. Mol Pain 2011;7:91.
[10] Fang H, Zhang HH, Yang BX, Huang JL, Shun JL, Kong FJ, Peng X, Chen ZG, Lu JM. Cdk5 contributes to inflammation-induced thermal hyperalgesia mediated by the p38 MAPK pathway in microglia. Brain Res 2015;1619:166–75.
[11] Franceschini A, Vilotti S, Ferrari M, van den Maagdenberg AMJ, Nistri A, Fabbretti E. TNFα levels and macrophages expression reflect an inflammatory potential of trigeminal ganglia in a mouse model of familial hemiplegic migraine. PLoS One 2013;8:e52394.
[12] Hall BE, Zhang L, Sun ZJ, Utreras E, Prochazkova M, Cho A, Terse A, Arany P, Dolan JC, Schmidt BL, Kulkarni AB. Conditional TNF-alpha overexpression in the tooth and alveolar bone results in painful pulpitis and osteitis. J Dent Res 2015;95:188–95.
[13] Hall BE, Zheng C, Swaim WD, Cho A, Nagineni CN, Eckhaus MA, Flanders KC, Ambudkar IS, Baum BJ, Kulkarni AB. Conditional overexpression of TGF-beta1 disrupts mouse salivary gland development and function. Lab invest 2010;90:543–55.
[14] Harada T, Morooka T, Ogawa S, Nishida E. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nat Cell Biol 2001;3: 453–9.
[15] Hargreaves KM. Orofacial pain. PAIN 2011;152(3 suppl):S25–32.
[16] Hensellek S, Brell P, Schaible HG, Brauer R, Segond von Banchet G. The cytokine TNFalpha increases the proportion of DRG neurones expressing the TRPV1 receptor via the TNFR1 receptor and ERK activation. Mol Cell Neurosci 2007;36:381–91.
[17] Kawauchi T. Cdk5 regulates multiple cellular events in neural development, function and disease. Dev Growth Differ 2014;56:335–48.
[18] Khan AA, Diogenes A, Jeske NA, Henry MA, Akopian A, Hargreaves KM. Tumor necrosis factor alpha enhances the sensitivity of rat trigeminal neurons to capsaicin. Neuroscience 2008;155:503–9.
[19] Kuner R. Central mechanisms of pathological pain. Nat Med 2010;16: 1258–66.
[20] Kurokouchi K, Kambe F, Yasukawa K, Izumi R, Ishiguro N, Iwata H, Seo H. TNF-alpha increases expression of IL-6 and ICAM-1 genes through activation of NF-kappaB in osteoblast-like ROS17/2.8 cells. J Bone Miner Res 1998;13:1290–9.
[21] Langert K, Von Zee C, Stubbs E. Tumour necrosis factor α enhances CCL2 and ICAM-1 expression in peripheral nerve microvascular endoneurial endothelial cells. ASN Neuro 2013;5:e00104.
[22] Lee KM, Jeon SM, Cho HJ. Tumor necrosis factor receptor 1 induces interleukin-6 upregulation through NF-kappaB in a rat neuropathic pain model. Eur J Pain 2009;13:794–806.
[23] Lei L, Yuan X, Wang S, Zhang F, Han Y, Ning Q, Luo G, Lu S. Mitogen-activated protein kinase pathways are involved in the upregulation of calcitonin gene-related peptide of rat trigeminal ganglion after organ culture. J Mol Neurosci 2012;48:53–65.
[24] Leung L, Cahill CM. TNF-alpha and neuropathic pain–a review. J Neuroinflammation 2010;7:27.
[25] Li K, Zhao GQ, Li LY, Wu GZ, Cui SS. Epigenetic upregulation of Cdk5 in the dorsal horn contributes to neuropathic pain in rats. Neuroreport 2014; 25:1116–21.
[26] Liu J, Du J, Yang Y, Wang Y. Phosphorylation of TRPV1 by cyclin-dependent kinase 5 promotes TRPV1 surface localization, leading to inflammatory thermal hyperalgesia. Exp Neurol 2015;273:253–62.
[27] Liu X, Liu Y, Zhang J, Zhang W, Sun YE, Gu X, Ma Z. Intrathecal administration of roscovitine prevents remifentanil-induced postoperative hyperalgesia and decreases the phosphorylation of N-methyl-D-aspartate receptor and metabotropic glutamate receptor 5 in spinal cord. Brain Res Bull 2014;106:9–16.
[28] Madrid R, de la Pena E, Donovan-Rodriguez T, Belmonte C, Viana F. Variable threshold of trigeminal cold-thermosensitive neurons is determined by a balance between TRPM8 and Kv1 potassium channels. J Neurosci 2009;29:3120–31.
[29] Mamet J, Klukinov M, Yaksh TL, Malkmus SA, Williams S, Harris S, Manning DC, Taylor BK, Donahue RR, Porreca F, Xie JY, Oyarzo J, Brennan TJ, Subieta A, Schmidt WK, Yeomans DC. Single intrathecal administration of the transcription factor decoy AYX1 prevents acute and chronic pain after incisional, inflammatory, or neuropathic injury. PAIN 2014;155:322–33.
[30] Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B. Importance of glial activation in neuropathic pain. Eur J Pharmacol 2013; 716:106–19.
[31] Moalem G, Tracey D. Immune and inflammatory mechanisms in neuropathic pain. Brain Res Rev 2006;51:240–64.
[32] Nair A, Simonetti M, Fabbretti E, Nistri A. The Cdk5 kinase downregulates ATP-gated ionotropic P2X3 receptor function via serine phosphorylation. Cell Mol Neurobiol 2010;30:505–9.
[33] Neubert JK, Widmer CG, Malphurs W, Rossi HL, Vierck CJ Jr, Caudle RM. Use of a novel thermal operant behavioral assay for characterization of orofacial pain sensitivity. PAIN 2005;116:386–95.
[34] Pareek TK, Keller J, Kesavapany S, Agarwal N, Kuner R, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB. Cyclin-dependent kinase 5 modulates nociceptive signaling through direct phosphorylation of transient receptor potential vanilloid 1. Proc Natl Acad Sci U S A 2007; 104:660–5.
[35] Pareek TK, Keller J, Kesavapany S, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB. Cyclin-dependent kinase 5 activity regulates pain signaling. Proc Natl Acad Sci U S A 2006;103:791–6.
[36] Pareek TK, Kulkarni AB. Cdk5: a new player in pain signaling. Cell Cycle 2006;5:585–8.
[37] Pertusa M, Gonzalez A, Hardy P, Madrid R, Viana F. Bidirectional modulation of thermal and chemical sensitivity of TRPM8 channels by the initial region of the N-terminal domain. J Biol Chem 2014;289: 21828–43.
[38] Prochazkova M, Terse A, Amin ND, Hall B, Utreras E, Pant HC, Kulkarni AB. Activation of cyclin-dependent kinase 5 mediates orofacial mechanical hyperalgesia. Mol Pain 2013;9:66.
[39] Reid G, Amuzescu B, Zech E, Flonta ML. A system for applying rapid warming or cooling stimuli to cells during patch clamp recording or ion imaging. J Neurosci Methods 2001;111:1–8.
[40] Sauer B, Henderson N. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol 1990;2:441–9.
[41] Sessle BJ. Peripheral and central mechanisms of orofacial inflammatory pain. Int Rev Neurobiol 2011;97:179–206.
[42] Shah K, Lahiri DK. Cdk5 activity in the brain—multiple paths of regulation. J Cell Sci 2014;127(pt 11):2391–400.
[43] Sommer C, Kress M. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett 2004;361:184–7.
[44] Stamatovic SM, Keep RF, Kunkel SL, Andjelkovic AV. Potential role of MCP-1 in endothelial cell tight junction "opening": signaling via Rho and Rho kinase. J Cell Sci 2003;116(pt 22):4615–28.
[45] Takahashi N, Kikuchi S, Shubayev VI, Campana WM, Myers RR. TNF-alpha and phosphorylation of ERK in DRG and spinal cord: insights into mechanisms of sciatica. Spine (Phila Pa 1976) 2006;31:523–9.
[46] Takahashi S, Ohshima T, Cho A, Sreenath T, Iadarola MJ, Pant HC, Kim Y, Nairn AC, Brady RO, Greengard P, Kulkarni AB. Increased activity of cyclin-dependent kinase 5 leads to attenuation of cocaine-mediated dopamine signaling. Proc Natl Acad Sci U S A 2005;102:1737–42.
[47] Takeda M, Takahashi M, Matsumoto S. Contribution of the activation of satellite glia in sensory ganglia to pathological pain. Neurosci Biobehav Rev 2009;33:784–92.
[48] Utreras E, Futatsugi A, Pareek TK, Kulkarni AB. Molecular Roles of Cdk5 in Pain Signaling. Drug Discov Today Ther Strateg 2009;6:105–11.
[49] Utreras E, Futatsugi A, Rudrabhatla P, Keller J, Iadarola MJ, Pant HC, Kulkarni AB. Tumor necrosis factor-alpha regulates cyclin-dependent kinase 5 activity during pain signaling through transcriptional activation of p35. J Biol Chem 2009;284:2275–84.
[50] Utreras E, Hamada R, Prochazkova M, Terse A, Takahashi S, Ohshima T, Kulkarni AB. Suppression of neuroinflammation in forebrain-specific Cdk5 conditional knockout mice by PPARgamma agonist improves neuronal loss and early lethality. J Neuroinflammation 2014;11:28.
[51] Utreras E, Keller J, Terse A, Prochazkova M, Iadarola MJ, Kulkarni AB. Transforming growth factor-beta1 regulates Cdk5 activity in primary sensory neurons. J Biol Chem 2012;287:16917–29.
[52] Utreras E, Maccioni R, Gonzalez-Billault C. Cyclin-dependent kinase 5 activator p35 over-expression and amyloid beta synergism increase apoptosis in cultured neuronal cells. Neuroscience 2009;161:978–87.
[53] Utreras E, Prochazkova M, Terse A, Gross J, Keller J, Iadarola MJ, Kulkarni AB. TGF-beta1 sensitizes TRPV1 through Cdk5 signaling in odontoblast-like cells. Mol Pain 2013;9:24.
[54] Utreras E, Terse A, Keller J, Iadarola MJ, Kulkarni AB. Resveratrol inhibits Cdk5 activity through regulation of p35 expression. Mol Pain 2011;7:49.
[55] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45–65.

[56] Wang GQ, Cen C, Li C, Cao S, Wang N, Zhou Z, Liu XM, Xu Y, Tian NX, Zhang Y, Wang J, Wang LP, Wang Y. Deactivation of excitatory neurons in the prelimbic cortex via Cdk5 promotes pain sensation and anxiety. Nat Commun 2015;6:7660.
[57] Wang X, de Rivero Vaccari JP, Wang H, Diaz P, German R, Marcillo AE, Keane RW. Activation of the nuclear factor E2-related factor 2/antioxidant response element pathway is neuroprotective after spinal cord injury. J Neurotrauma 2012;29:936–45.
[58] Weber KS, Nelson PJ, Grone HJ, Weber C. Expression of CCR2 by endothelial cells: implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium. Arteriosclerosis Thromb Vasc Biol 1999;19:2085–93.
[59] White FA, Bhangoo SK, Miller RJ. Chemokines: integrators of pain and inflammation. Nat Rev Drug Discov 2005;4:834–44.
[60] Xie WY, He Y, Yang YR, Li YF, Kang K, Xing BM, Wang Y. Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the delta-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. J Neurosci 2009;29:3551–64.
[61] Xing BM, Yang YR, Du JX, Chen HJ, Qi C, Huang ZH, Zhang Y, Wang Y. Cyclin-dependent kinase 5 controls TRPV1 membrane trafficking and the heat sensitivity of nociceptors through KIF13B. J Neurosci 2012;32: 14709–21.
[62] Xu XJ, Tang YM, Song H, Yang SL, Xu WQ, Shi SW, Zhao N, Liao C. A multiplex cytokine score for the prediction of disease severity in pediatric hematology/oncology patients with septic shock. Cytokine 2013;64:590–6.
[63] Xu ZZ, Zhang L, Liu T, Park J, Berta T, Yang R, Serhan C, Ji RR. Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. Nat Med 2010;16:592.
[64] Yamakawa I, Kojima H, Terashima T, Katagi M, Oi J, Urabe H, Sanada M, Kawai H, Chan L, Yasuda H, Maegawa H, Kimura H. Inactivation of TNF-alpha ameliorates diabetic neuropathy in mice. Am J Physiol Endocrinol Metab 2011;301:E844–852.
[65] Yang L, Gu X, Zhang W, Zhang J, Ma Z. Cdk5 inhibitor roscovitine alleviates neuropathic pain in the dorsal root ganglia by downregulating N-methyl-D-aspartate receptor subunit 2A. Neurol Sci 2014;35: 1365–71.
[66] Yang YR, He Y, Zhang Y, Li Y, Han Y, Zhu H, Wang Y. Activation of cyclin-dependent kinase 5 (Cdk5) in primary sensory and dorsal horn neurons by peripheral inflammation contributes to heat hyperalgesia. PAIN 2007; 127:109–20.
[67] Yang Y, Wang H, Dou Y, Wang Y, Han G, Wang R, Wang L, Guo R, Xiao H, Li X, Shen B, Shi Y, Chen G, Li Y. Colitogenic role of tumour necrosis factor (TNF) receptors in trinitrobenzene sulphonic acid colitis: TNF-R1 ablation does not affect systemic inflammatory response. Clin Exp Immunol 2011;165:372–82.
[68] Zelenka M, Schafers M, Sommer C. Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. PAIN 2005;116: 257–63.
[69] Zhang Q, Yu J, Wang J, Ding CP, Han SP, Zeng XY, Wang JY. The red nucleus TNF-alpha participates in the initiation and maintenance of neuropathic pain through different signaling pathways. Neurochem Res 2015;40:1360–71.
[70] Zhang X-C, Kainz V, Burstein R, Levy D. Tumor necrosis factor-α induces sensitization of meningeal nociceptors mediated via local COX and p38 MAP kinase actions. PAIN 2011;152:140–9.
[71] Zhang X, Zhang H, Shao H, Xue Q, Yu B. ERK MAP kinase activation in spinal cord regulates phosphorylation of Cdk5 at serine 159 and contributes to peripheral inflammation induced pain/hypersensitivity. PloS One 2014;9:e87788.