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Targeted overexpression of Tumor Necrosis Factor- α increases Cyclin-dependent kinase 5 activity and TRPV1-dependent Ca^{2+} influx in trigeminal neurons

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Abstract

We reported earlier that TNF- α , a proinflammatory cytokine implicated in many inflammatory disorders causing orofacial pain increases Cdk5 activity, a key kinase involved in brain development and function and recently in pain signaling. To investigate a potential mechanism underlying inflammatory pain in trigeminal ganglia (TG), 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 NF κ B 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-evoked Ca^{2+} influx in cultured trigeminal neurons. Remarkably, this effect was prevented by roscovitine, an inhibitor of Cdk5, suggesting 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

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Conflict of Interest Statement

The authors had no conflicts of interest to declare.

sensory neurons of TNF- α cTg mice results in inflammatory sensitization and increased Cdk5 activity, therefore this mouse model would be valuable for investigating mechanism involved TNF- α in orofacial pain.

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 while 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's Adjuvant [67]. 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]. Cdk5 activity results in an increased of agonist-induced Ca^{2+} influx in cultured dorsal root ganglia (DRG) [34] and 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 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, as well as 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 trigeminal ganglia, TNF- α increases the sensitivity of TRPV1+ neurons to capsaicin in part by sustained up-regulation of TRPV1 expression [18].

In the present 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 trigeminal ganglia, implicating its potential role in orofacial pain.

Methods

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 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 (Figure 1A). The pCLE-TNF- α vector was then transformed into DH5- α competent *E. coli* cells and positive clones were screened by PCR using TNF- α primers and confirmed by DNA sequencing.

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 h. The supernatants were collected and stored at -80°C for subsequent TNF- α ELISA assays. Protein and total RNA were obtained for Western blot and 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 co-transfected pCLE-TNF- α and CMV-Cre vectors transiently into PC12 clone C7 (stably transfected with p35 promoter-luciferase vector) for 24 h [54]. After that, the proteins were extracted from the treated cells, and p35 promoter-driven luciferase activity was measured by 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.

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 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.

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). PCR for GFP was

performed for 35 cycles consisting of 30 s at 94°C, 1 min at 60°C, and 1 min 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. PCR 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 min at 94°C, 30 s at 60°C, and 1 min at 72°C. All PCR products were electrophoresed on 2% agarose gels in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.4) and stained with Ethidium bromide (1 μ g/mL).

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

All mice were euthanized with a lethal dose of 10% Ketamine-2% Xylazine (100 μ L/each) injected i.p. 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 versus age and the mean values were compared between the two genotypes and genders. Blood samples were collected by cardiac puncture in 1 and 3 month old mice. Blood samples were incubated for 1 h at room temperature followed by 5 min on ice and centrifuged at 3500 rpm for 20 min 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.

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 h followed by incubation in a sucrose solution (30%) for an additional 24 h. In order to avoid decrease in endogenous GFP fluorescence, some TGs from control and TNF- α cTg mice were fixed for 20 min in Zamboni's 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 h. TGs were then embedded in OCT (Optimal Cutting Temperature from Sakura Finetek, Tokyo, Japan), and sliced into 14 μ m 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 albumine (BSA)) for 1 h at room temperature followed by incubation with primary antibody in 1% BSA in phosphate-buffered saline (BSA-PBS) overnight at 4°C. TG sections were then incubated with secondary antibodies (anti-rabbit, anti-mouse, 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 h 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, Jena, Germany) and processed with the LSM Image Browser (Carl Zeiss Microscopy) software. Immunofluorescence of TG sections and cultured trigeminal neurons were performed using the following antibodies: anti- β III tubulin mouse antibody clone G7121 from Promega (Madison, WI) as neuronal markers, ionized calcium-binding adapter molecule 1 (Iba1) rabbit antibody #019-19741 from Wako (Richmond, VA) as macrophage marker, glial fibrillary acidic protein (GFAP) mouse antibody #G3893 and glutamine synthetase (GS)

rabbit antibody #G2781 from Sigma-Aldrich (San Louis, MO) as satellite glial cell markers, phospho-p44/p42 MAPK (ERK1/2) rabbit antibody #9101, phospho-NF κ B 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 (San Louis, MO) was used as non-peptidergic 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 the LSM Image Browser software (Carl Zeiss Microscopy) and ImageJ software (NIH, Bethesda MD, USA). 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.

Preparation of RNA and RT-PCR

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, Carlsbad, CA), according to the manufacturer's instructions. Following 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 μ g of total RNA was made using Super-ScriptTM III reverse transcriptase (Invitrogen, Carlsbad, CA). We analyzed the mRNA expression of TNF- α , interleukin 12 (IL-12), interferon γ (IFN- γ), Monocyte chemo-attractant protein-1 (MCP-1), IL-6, interleukin 1 β (IL-1 β), interleukin 10 (IL-10), transforming growth factor- β 1 (TGF- β 1), 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.

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, Carlsbad, CA). The membranes were soaked in blocking buffer (5% nonfat dry milk in PBS with 0.05% Tween-20 (PBST)) for 1 h 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 h at room temperature with the secondary antibodies diluted in blocking buffer. Immunoreactivity was detected by using Super-Signal West Pico or Dura

Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). For semi-quantitative 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, Bethesda, MD). Western blot from TG tissues were performed for Cdk5 using rabbit antibody C8 (Santa Cruz Biotechnology, Dallas, TX) and p35 rabbit antibody C64B10 (Cell Signaling, Danvers, MA) and α -tubulin antibody from Sigma-Aldrich (San Louis, MO) as loading control.

Cdk5 kinase activity assay

200 μ g of protein from TG of 3-month old TNF- α cTg and control mice was dissolved in T-PER buffer and immunoprecipitated using 4 μ g of anti-Cdk5 (C8) antibody (Santa Cruz Biotechnology, Dallas, TX). Briefly, immunoprecipitated proteins (IP) were washed three times in cold PBS, and twice in kinase buffer (20 mM Tris HCl (pH 7.4), 10 mM MgCl₂, 1 mM EDTA). Then, the IP was mixed with the kinase assay mixture (100 mM Tris HCl (pH 7.4), 50 mM MgCl₂, 5 mM EDTA, and 5 mM DTT) plus 5 units of (γ P³²)-ATP, and 5 μ g of histone H1 as a substrate. The kinase assays were carried out for 45 minutes at 30°C, and the kinase activity reaction was stopped by adding loading buffer and boiling for 10 min 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 P³² to histone H1 was quantified by measuring band intensity using an image analysis system with ImageJ 1.46r software.

Culture of trigeminal ganglia 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, San Louis, MO) in a INC-mix solution (NaCl 155 mM; K₂HPO₄ 1.5 mM; HEPES 10 mM; glucose 5 mM; at pH 7,4). The enzymatic digestion was performed for 45 min at 37°C in 5% CO₂, and cells were cultured in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), Pen/Strep 100 μ g/mL, MEM-vit (Invitrogen, Carlsbad, CA), and nerve growth factor (100 ng/ml; Sigma-Aldrich, San Louis, MO). Cells were plated on 6 or 13 mm poly-l-lysine-coated glass coverslips and used after 4 h for Ca²⁺ imaging analysis or after 18 h for immunofluorescence analysis. In some cases, roscovitine (10 μ M) was added for 4 h before Ca²⁺ imaging experiments.

HEK-293 cells culture and transfection

HEK-293 cells were plated in 24-well dishes at 1 $\times$ 10⁵ cells/well, and transiently transfected with 1 μ g of the indicated DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions (see [37]). At 48 h post-transfection, Ca²⁺ imaging analysis was performed.

Ca²⁺ imaging

Trigeminal neurons and HEK293 cells were incubated with 5 μ M Fura-2 AM dissolved in standard extracellular solution and 0.02% Pluronic (Invitrogen, Carlsbad, CA) for 45–60 min at 37°C. Fluorescence measurements were made with a Nikon Ti inverted microscope

fitted with a cooled digital CCD camera (Orca-03G, Hamamatsu, 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. 340/380 ratios (0.5 Hz) were displayed online with HCAImage 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) supplemented with an IT-18 T-thermocouple and digitized with an Axon Digidata 1440A AD converter running Clampex 10 software (Molecular Devices).

Control of temperature and chemical stimuli

Coverslips with cultured TG neurons were placed in a microchamber and continuously perfused with extracellular solution warmed at $34\pm 1^{\circ}\text{C}$. 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 showed 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 [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 three different concentrations (2, 20 and 200 nM) in cells transfected with TRPV1-GFP and co-transfected with pcDNA3 (control) and p35 in pcDNA3 (p35) respectively.

Orofacial pain behavior test

An orofacial pain test was performed as previously described [2; 33]. Briefly, control littermates and TNF- α cTg mice of 6–8 month old were trained on an 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 h before receiving training. Firstly, we trained control and TNF- α mice in the operant pain assessment device (OPAD) apparatus for 3–4 times at a non-aversive temperature (33°C). Next week, we used a 20 min 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 (non-aversive temperature) during 3 min followed by heated to 45°C over 30 sec and the keeping at 45°C (aversive temperature) for 3 min more. The numbers 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 of less than 0.05 was considered significant.

Statistical Analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism software v5.0. Significant differences between experiments were assessed by an unpaired Student's t-test where α was set to 0.05. For comparisons between control and TNF- α cTg mice at different ages, we used a two way ANOVA with post-hoc tests. For Ca^{2+} imaging experiments, we used Fisher's test to compare the percentage of trigeminal neurons responding to capsaicin.

Results

Generation of TNF^{glo} mice

In order 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 (Figure 1A). Prior to 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 , while this expression was considerably decreased in cells co-transfected with pCLE-TNF- α and CMV-Cre vectors, indicating Cre-mediated deletion of EGFP (Figure 1B). TNF- α mRNA levels were also significantly increased in these co-transfected cells as compared to the cells transfected only with pCLE-TNF- α or untransfected cells, as measured by qPCR (Figure 1C). Furthermore, our Western blot analyses confirmed that EGFP protein expression was higher in cells transfected with pCLE-TNF- α (relative expression=1.0), while it was decreased in cells co-transfected 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 co-transfected with pCLE-TNF- α and CMV-Cre vectors as compared to the cells transfected only with pCLE-TNF- α or untransfected cells (Figure 1D). However, we were unable to detect soluble TNF- α (17 kDa) in these cell extracts. Whereas, 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 co-transfected with both vectors as compared with the cells transfected only with pCLE-TNF- α vector or the untransfected cells (Figure 1E). Finally, in order 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 if the secreted TNF- α increased p35 promoter activity [49; 54]. To carry out this analysis, C7 cells were transiently transfected with pCLE-TNF- α and CMV-Cre vectors and after 24 h, 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 (Figure 1F). As a positive control, we transfected CMV-TNF- α vector which overexpressed high levels of TNF- α as detected by ELISA (data not shown), into C7 cells and found that p35 promoter-luciferase activity was significantly increased (Figure 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 three recipient females that were transplanted with the injected zygotes. We identified three founder lines (B1, B11 and C10) based on visual inspection for GFP using the Macro Imaging System (Figure 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) (Figure 2B). Then, we expanded C10 founder line which showed higher expression of GFP, breeding it with wild type mice. Next, we did immunofluorescence against β III-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 (Figure 2C). Phenotypic analysis of transgenic mice demonstrated that they were born healthy and viable, without any signs of adverse effects of transgene integration.

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 (Figure 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 β III-tubulin (red) and endogenous GFP fluorescence (green) we found that some neurons (white arrow) were GFP negative indicating removal of GFP by Cre recombination (Figure 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 old 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.

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 (Figure 3A) and protein (Figure 3B) levels. Using immunofluorescence analysis, we found that TNF- α was expressed at detectable levels in trigeminal neurons of the control mice (Figure 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 (Figure 3C and E). We next evaluated what type of cells expressed TNF- α in these mice. First, we detected peptidergic neurons by using CGRP immunostaining (yellow arrow) and non peptidergic neurons by using IB4 immunostaining of TG sections of TNF- α cTg mice (Figure 3F). We found that TNF- α is highly expressed in all types of neurons. In particular, TNF- α co-localized with IB4 positive neurons and with also others neurons, possibly TRPA1 and CGRP positive neurons in TG from TNF- α cTg mice (Figure 3F–H). TNF- α plays a pivotal role during neuro-immune interaction through activation of NF κ B signaling pathway [24; 55; 64]. Interestingly, we found that TNF- α

overexpression resulted in a significantly increased staining of phospho-NF κ B p65 in TGs of TNF- α cTg mice as compared with control mice of 3 months of age (Figure 3I) suggesting that TNF- α triggers activation of NF κ B signaling *in vivo*.

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 glia cells in TG from TNF- α cTg and control mice by using Iba1 (Figure 4A) and GFAP (Figure 4B) markers, respectively. We found that TG sections of TNF- α cTg mice had significantly increased staining of Iba1 in cells surrounding trigeminal neurons (Figure 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 (Figure 4B), suggesting that TNF- α overexpression results in local inflammation in nociceptive tissues. Secondly, 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-1 β , IL-10, TGF- β 1, LIF, OSM and TNF- α , in TG and brain of TNF- α cTg and control mice (Figure 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 (Figure 4C, upper panel). In addition, we confirmed significantly increase in IL-6 levels by immunofluorescence staining in TG of TNF- α cTg mice (Figure 4D). Interestingly, there was no difference in mRNA levels of all cytokines assayed in brains of these mice (Figure 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 to controls (6.3 ± 1.7 pg/mL) (Figure 5A) although such increased level was within the reported physiological range for TNF- α levels [66]. 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 six 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 to littermate controls (Figure 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.

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, Figure 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, Figure 6C) at 3 and 6 months of age in TG from TNF- α cTg mice as compared with controls (Figure 6C and D).

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

Since 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 (Figure 7A and B) and p35 immunostaining (Figure 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 (Figure 7E). However, p35 protein level was significantly increased in TG from TNF- α cTg as compared with control mice at 3-month old (Figure 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-month olds (Figure 7G), indicating that TNF- α overexpression increases Cdk5 kinase activity *in vivo*, possibly by an increase in p35 levels.

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 Ca²⁺ influx in cultured DRG neurons and odontoblast-like cells [34; 51; 53]. Therefore, we assessed if increased Cdk5 activity in TNF- α cTg mice elevates phosphorylation of TRPV1 at T407 in TG as well as 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 (Figure 8A and B). However, we found significant increase in phospho-T407-TRPV1 in TG of TNF- α cTg mice as compared with control mice at 3-month old (Figure 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 h and then we performed immunofluorescence for phospho-T407-TRPV1 (blue) and β III-tubulin (red) in these cultures (Figure 8E). Interestingly, we found a significant increase in phospho-T407-TRPV1 staining in TG neurons, that were GFP negative (see white arrows), from TNF- α cTg mice as compared with control mice (Figure 8F), suggesting that Cdk5 activity can regulate phosphorylation of TRPV1 in TG tissue and cultured TG neurons.

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 Ca²⁺ imaging experiments in the heterologous system of HEK-293 cells co-transfected 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 to control cells (Figure 9A and B). Moreover, 49% (53/108) of the cells responding to 200 nM capsaicin also respond to 2 nM capsaicin in p35(+) cells, while only 32% (30/94) of control cells show sensitivity to this subsaturating concentration of the vanilloid ($p < 0.05$, *F* test) (Figure 9A and B). On the other hand, the responses to 20 nM and 200 nM are similar, indicating 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].

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 h after plating) from TNF- α cTg and control mice, treated with capsaicin and finally with KCl as a control of viability (Figure 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 to control mice (10.5%) (Figure 9D). Then, we performed the same experiment in the presence or absence of the Cdk5 inhibitor roscovitine (10 μM) that was present during 4 h 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, pre-treatment of TG cultures from TNF- α cTg with roscovitine (10 μM) prevented the increase in the percentage of capsaicin-responsive neurons from the untreated group (from 35.1% to 6.7%) suggesting that Cdk5 regulates the TRPV1-dependent Ca^{2+} influx by TRPV1 phosphorylation (Figure 9D). Finally, we did not find change in the amplitude of capsaicin-evoked $[\text{Ca}^{2+}]_i$ elevations at 20 nM in both genotypes (data not shown).

TNF- α overexpression in TG increased orofacial thermal pain sensation

We performed operant behavioral tests on TNF- α cTg and control mice of 6–8 months old by using the OPAD, previously used to measure orofacial thermal pain in rodents [2]. After 15 ± 1 h of water and food withdrawal, we measured reward licking events (number of licks) at two temperatures: 33°C (non-aversive) during 3 min followed by 45°C (aversive) during 3 min 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 (Figure 10A), the lick-to-face contact ratio (Figure 10B), and total water intake (Figure 10C) between control and TNF- α cTg mice during a 20 min test at 45°C (aversive temperature). We found a significant reduction of all these parameters on TNF- α cTg mice as compared with control mice indicating the important role of TNF- α in orofacial thermal sensitivity.

Discussion

We and others discovered that Cdk5 plays an important role during inflammation-induced pain signaling [36; 38; 48; 67]. Moreover, we identified the relevance of cytokines to Cdk5 activation by showing that TNF- α and TGF- β 1 increase Cdk5 activity through ERK1/2 signaling, which in turn increases Egr1 and p35 expression in PC12 cells, odontoblast-like cells, and in 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 rat, where 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 appears to be dependent on Cdk5 activity [10; 25]. Interestingly, knocking down Cdk5 in the prefrontal 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 Cdk5/p35 system's involvement 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 supports 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], P2 $\times$ 3 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 Ca²⁺ 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 the current study, we set out to determine whether TNF- α modulates Cdk5 activity *in vivo*. Current mouse models that have been developed to understand the role of TNF- α in inflammatory pain utilize traditional approaches such as administering CFA, carrageenan or formalin into the paws, or TNF- α administration at intrathecal, subcutaneous or at cranial meninges sites [11; 31; 63; 68; 70]. However, these models proved to be transient with a considerable variation over a period of time. 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- β 1^{glo} mice [13], and recently TNF- α ^{glo} mice in 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-diameters 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 NF κ B 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 MCP1, 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, MCP1, and Iba1 were observed in the TG from a migraine mouse model suggesting that TNF- α might modulate sensory neurons and resident glia cells, underlying the process of neuronal sensitization [11]. Nevertheless, in this migraine mouse model, IL-10 and IL-1 β mRNA were also upregulated, indicating that different mechanisms could be operating in these different genetic approaches. TNF- α can increase IL-6 expression through NF κ B activation in osteoclast-like cells [20] and also it can increase MCP1 levels in endothelial cells [21]. Furthermore, TNFR1 antisense oligonucleotides can decrease IL-6 regulation through NF κ B signaling in a rat neuropathic pain model [22]. On the other hand, 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 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, co-expression with p35 resulted in an increase of the responses of the cells to a subsaturating concentration of this vanilloid, compared to 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 reached calcium entry due to the activation of voltage-gated Ca²⁺ 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 patch clamp technique, in order 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 pro-inflammatory 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-1 β [47]. Another cell population participating in TG environment is vascular endothelial cells. These cells respond to MCP1 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 non-autonomous 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 non-aggressive phenotype for TNF- α overexpression. This long lasting TNF- α expression can be a desirable feature for studying chronic pain states where several inflammatory components are deregulated (reviewed in [19]). Since the Nav1.8 promoter is also expressed in sensory neurons of the DRG, we expect our findings related to trigeminal-mediated pain can be apply to the dorsal root ganglia and dorsal horn of spinal cord. Therefore, our TNF- α cTg mouse model will be a valuable tool to study molecular mechanisms of inflammatory pain.

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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 h. 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 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 h. Relative intensity of 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 h measured by TNF- α ELISA. 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 h and p35 promoter luciferase activity was measured by a luciferase kit. The bars graph represent mean $\pm$ SEM. (n=3–6 samples) *, p<0.05 and ***, p<0.005 as compared to no transfection.

Figure 2.

Generation of TNF- α cTg mice. A) Representative image of two mice at P4 age, one TNF^{glo} mouse displays endogenous GFP fluorescence (green) whereas 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 to 4.6 kb was detected only in founder C10 mice. C) Representative immunofluorescence of TG from TNF- α mice at 3 months old showing endogenous GFP fluorescence (green), β III-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 old showing endogenous GFP fluorescence (green), β III-tubulin antibody (neurons in red), and TO-PRO-3 (nuclei in blue). GFP expression is absent in some neurons where Cre recombination occurred driven by Nav1.8 promoter (white arrow).

Figure 3.

TNF-α overexpression in TG from TNF-α cTg mice. A) Relative TNF-α mRNA levels in TG from TNF-α cTg and control mice at 3 months old (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 old measured by ELISA (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 old (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 old. White arrow showed Cre recombination and overexpression of TNF- α immunodetection. Representative immunofluorescence of: F) CGRP (red) and IB4 (green); (G) TRPA1 (red) and β III-tubulin; and H) TNF- α (red) and IB4 (green) of TG from TNF- α cTg mice at 3 months old. (Bar=50 μ m). I) Representative immunofluorescence of phospho-p65 (P-p65, green) of TG from TNF- α cTg and control mice at 3 months old and the quantification of P-p65 immunodetection (n=2 mice, between 22–30 neurons). The bar graph represents mean $\pm$ SEM. *, p<0.05, **, p<0.01 and ***, p<0.005 as compared to control mice.

Figure 4.

TNF-α overexpression caused inflammation in TG 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 old (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 old (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 old. 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 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 bars graph represents mean ± SEM. *, p<0.05, **, p<0.01 and ***, p<0.005 as compared to control mice.

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 old measured by TNF- α ELISA (n=5–6 mice). B) Quantification of the expression of six inflammatory mediators (IL-12, TNF- α , IFN- γ , MCP-1, IL-6, and IL-10) in serum from TNF- α cTg and control mice at 3 months old by using Cytometric Bead Array (CBA). IL-10 was not detected (n=4–6 mice). The bars graph represent mean $\pm$ SEM. *, p<0.05 as compared to control mice.

Figure 6.

TNF- α overexpression activates ERK1/2 pathway and increases Egr1 expression in TG 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 old. 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 old. (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 old. 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 old. (n=2 mice, between 20–21 neurons). (Bar=50 μ m). The bars graph represents mean $\pm$ SEM. ***,p<0.001 as compared to control mice (two way-ANOVA, with Bonferroni posttest).

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Figure 7.

TNF-α overexpression increases Cdk5 and p35 expression and Cdk5 kinase activity in TG 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 old (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 old (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 old (n=2 mice, between 34–42 neurons). The bars graph represent mean $\pm$ SEM. *, p<0.05, **, p<0.01 and ***, p<0.005 as compared to control littermate mice (two way-ANOVA two 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 old. 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 graph represents mean $\pm$ SEM. *, p<0.05, as compared to control littermate mice (Student's t-test).

Figure 8.

TNF- α overexpression increases Cdk5-mediated TRPV1 phosphorylation at T407 in TG 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 old (bar=50 μ m). B) Quantification of TRPV1 immunodetection in TG from TNF- α cTg mice as fold change compared with control mice at 3 months old (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 old (bar=50 μ m). D) Quantification of

phospho-T407-TRPV1 immunodetection in TG from TNF- α cTg mice as fold change compared with control mice at 3 months old (n=2 mice, between 36–43 neurons). E) Representative immunofluorescence of phospho-T407-TRPV1 (blue), β III-tubulin (red) and endogen GFP fluorescence (green) in cultured TG neurons from TNF- α cTg and control mice during 18 h. White arrow showed increase of phospho-T407-TRPV1 immunodetection in TG neurons from TNF- α cTg mice (bar=50 μ m). 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 bars graph represent mean $\pm$ SEM. *, p<0.05 and ***, p<0.001 as compared to control mice (Student's t-test).

Figure 9.

Cdk5/p35 potentiates TRPV1 sensitivity to capsaicin in HEK-293 cells and in cultured 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 co-expressed with p35 (red, right panel). B) Summary histogram of the results obtained for the experimental protocol in A. Statistical significance was assessed with a two-way ANOVA test. ratio of control condition (n=94) is different (***) 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 μ M), added 4 h before Ca^{2+} imaging experiments. (n=3–6 mice for each genotype, between 63–312 neurons analyzed). ***, $p<0.005$ as compared to control littermate mice (Fisher test). ###, $p<0.005$ as compared with TNF- α cTg mice (Fisher test).

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 min test at 45°C. The bars graphs represents mean $\pm$ SEM. (n=4–5 mice) *, p<0.05 as compared to control mice (Student's t-test).

Table 1

Sequence of primers used for genotyping in RT-PCR

Target	Primer sequences	Amplicon size (pb)
GFP	F: 5'-AAATTCATCTGCACCACCG-3'	173
	R: 5'-TCCTTGAAGAAGATGGTGCG-3'	
IC	F: 5'-CTAGGCCACAGAATTGAAAGATCT-3'	324
	R: 5'-GTAGGTGGAAATCTAGCATCATCC-3'	
Cre	F: 5'-GCACTGATTTGACCAGGTT-3'	421
	R: 5'-GAGTCATCCTTAGCGCCGTA-3'	
TNF- α	F: 5'-GATCTCAAAGACAACCACTAGT-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'-TCACCTGCTGCTACTCATTACCA-3'	250
	R: 5'-AAAGGTGCTGAAGACCCTAGGGCA-3'	
IL-6	F: 5'-TCCTCTCTGCAAGAGACTTCC-3'	546
	R: 5'-GCCACTCCTTCTGTGACTCC-3'	
IL-1 β	F: 5'-CAACCAACAAGTGATATTCTCCATG-3'	152
	R: 5'-GATCCACACTCTCCAGCTGCA-3'	
IL-10	F: 5'-ACTGGCATGAGGATCAGCAG-3'	351
	R: 5'-GAGAAATCGATGACAGCGCC-3'	
TGF- β 1	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'-GAAGGTCTGATTTGCGGGAT-3'	
S29	F: 5'-GGAGTCACCCACGGAAGTTCGG-3'	108
	R: 5'-GGAAGCACTGGCGGCACATG-3'	